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Atomic Weights

Symbol	Atomic Weight	Atomic Number	Symbol	Atomic Weight	Atom Numb
A	39.944	18	Mo	95.95	42
Ac	227	89	N	14.008	7
Ag	107.880	47	Na	22.997	11
Al	26.98	13	Nb	92.91	41
Am	[243]	95	Nd	144.27	60
As	74.91	33	Ne	20.183	10
At	[210]	85	Ni	58.69	28
Au	197.2	79	Np	[237]	93
B	10.82	5	O	16	8
Ba	137.36	56	Os	190.2	76
Be	9.013	4	P	30.975	15
Bi	209.00	83	Pa	231	91
Bk	[245]	97	Pb	207.21	82
Br	79.916	35	Pd	106.7	46
C	12.010	6	Pm	[145]	61
Ca	40.08	20	Po	210	84
Cd	112.41	48	Pr	140.92	59
Ce	140.13	58	Pt	195.23	78
Cf	[246]	98	Pu	[242]	94
Cl	35.457	17	Ra	226.05	88
Cm	[243]	96	Rb	85.48	37
Co	58.94	27	Re	186.31	75
Cr	52.01	24	Rh	102.91	45
Cs	132.91	55	Rn	222	86
Cu	63.54	29	Ru	101.7	44
Dy	162.46	66	S	32.066	16
Er	167.2	68	Sb	121.76	51
Eu	152.0	63	Sc	44.96	21
F	19.00	9	Se	78.96	34
Fe	55.85	26	Si	28.09	14
Fr	[223]	87	Sm	150.43	62
Ga	69.72	31	Sn	118.70	50
Gd	156.9	64	Sr	87.63	38
Ge	72.60	32	Ta	180.88	73
H	1.0080	1	Tb	159.2	65
He	4.003	2	Tc	[99]	43
Hf	178.6	72	Te	127.61	52
Hg	200.61	80	Th	232.12	90
Ho	164.94	67	Ti	47.90	22
I	126.91	53	Tl	204.39	81
In	114.76	49	Tm	169.4	69
Ir	193.1	77	U	238.07	92
K	39.100	19	V	50.95	23
Kr	83.80	36	W	183.92	74
La	138.92	57	Xe	131.3	54
Li	6.940	3	Y	88.92	39
Lu	174.99	71	Yb	173.04	70
Mg	24.32	12	Zn	65.38	30
Mn	54.93	25	Zr	91.22	40

Laboratory Manual of BIOCHEMISTRY

By

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Chemistry Department, City College of New York

Fourth Edition

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Preface to the Fourth Edition

As with the third edition, quite a number of changes have been made in this, the fourth edition. These changes are due to two causes: more and more experience with students in the laboratory and with their problems; and those advances in the field which can be put to use in a course in elementary biochemistry.

Aside from a number of reorganizations of experiments which were included in the third edition, specific additions in the present edition are the following: the time course of starch hydrolysis (page 5); filter paper chromatography (page 20); the estimation of catalase (page 42); an experiment on the fermentation of yeast (page 44); the estimation of gastric acidity (page 56); the absorption spectrum of hemoglobin and related compounds (page 76); the oxygen capacity of blood (page 115); several additions to the quantitative estimations of urinary constituents (pages 133 to 136); animal experiments (page 137); some vitamin estimations (page 140); and the details of a laboratory schedule for a two-semester course in biochemistry (page 142).

The Authors

New York City

Preface to the First Edition

THE PURPOSE of this Laboratory Manual is to provide simple experiments which will demonstrate certain phases of the subject matter covered in the author's "Textbook of Biochemistry." It was designed also for use with any of the standard textbooks on the subject. The Manual is the result of ten years' experience with students electing courses in biochemistry. Some of the points we wish to emphasize are the following:

1. The book, we believe, covers and more than covers the needs of students who wish to devote two semesters to laboratory work in biochemistry.

2. By appropriate selections, it is possible to offer an elementary introductory course in one semester.

3. Directions cannot be too precise. Every experiment in the Manual has been tested and retested; and the precise directions are the result of such experience. Even "qualitative" test-tube experiments are described in "quantitative" terms.

4. Under "Lipids" (Chapter 2) and "Proteins" (Chapter 3), the main operations deal with the isolation of products and, somewhat incidentally, with the identification of such products by appropriate test-tube experiments. Here the attempt has been made to apply the common procedure in organic chemistry that first calls for a specific preparation and then the appropriate tests for its identification.

5. The questions placed at the end of experiments should prove useful.

6. A number of experiments involving more recent procedures are included. Of these we may mention the application of photochemistry, the determination of sulfa drugs, and the uses of chromatographic analysis.

7. The use of "unknowns" in blood analyses.

8. Reagents for "qualitative" work will be found arranged alphabetically. Reagents for quantitative work are described as part of the specific experiment in question.

It is hoped that this Manual may be found useful in simplifying the laboratory work in biochemistry.

THE AUTHORS

New York City

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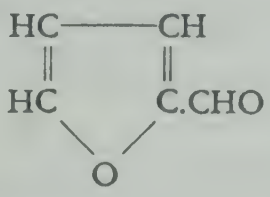
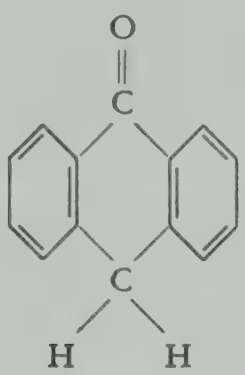
Chapter 1 **Carbohydrates**

(Details concerning the preparation of all reagents will be found in the Appendix, page 147.)

The Molisch test is a general test for carbohydrates. It is given by any compound which can be dehydrated to furfural,

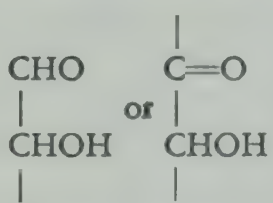


or a substituted furfural, by concentrated sulfuric acid. The color is due to various condensation products of furfural with α-naphthol. Another general test is the anthrone test. Anthrone,



which is the keto form of 9-hydroxy-anthracene, reacts with carbohydrates to produce a green to blue-green color.

Carbohydrates which have a free aldehyde or a ketone group are easily oxidized, thereby acting as reducing agents. The Benedict and Barfoed tests are based upon the reduction of Cu^{++} to Cu^{+}. Using the Barfoed test, it is possible to distinguish monosaccharides from disaccharides by controlling conditions (that is, *p*H and time of heating). Reducing sugars convert picric acid to picramic acid. Those carbohydrates which have the



groupings form osazones with phenylhydrazine.

There are several more or less specific reactions for certain carbohydrates.

For example, the Seliwanoff reaction is due to the conversion of fructose b hot hydrochloric acid into levulinic acid and hydroxymethylfurfural, and th condensation of the latter compound with resorcinol. The mucic acid test fo galactose takes advantage of the relative insolubility of mucic acid, which i formed by the oxidation of galactose. The iodine test may be used to dis tinguish starch from glycogen.

For experiments 1 to 9 use 1 per cent solutions of glucose, fructose galactose, maltose, lactose, sucrose and starch.

Experiment 1. Molisch Test. Add 2 drops of the Molisch reagent t 2 ml. of the sugar solution and mix thoroughly. Incline the tube, and pou down its side, slowly and carefully, 5 ml. of concentrated sulfuric acid.

(*a*) What is the color of the ring formed at the interface of the two liquids?

(*b*) What substance other than α-naphthol can be used as a reagent?

(*c*) What group of substances give the Molisch test?

(*d*) Why do many proteins give the Molisch test?

Experiment 2. Anthrone Test.* Add carefully 2 ml. of a 0.2 per cent solution of anthrone in concentrated sulfuric acid to 0.2 ml. of each of the carbohydrate solutions. Also add 2 ml. of a 0.2 per cent anthrone solution to a test tube containing some filter paper pulp. Shake each tube carefully and let it stand. Notice the color immediately and after a half hour. If a milkiness is produced, dilute carefully with glacial acetic acid or 50 per cent sulfuric acid. (Note: The final sulfuric acid concentration in the test solution should always be greater than 50 per cent.)

(*a*) Is there a change in intensity of the color upon standing?

(*b*) If the test solution contains other organic compounds besides carbohydrates, brown colors may be produced. Why?

Experiment 3. Picric Acid Test. Mix 2 ml. of each of the carbohydrate solutions with 1 ml. of saturated picric acid solution and 0.5 ml. of 1 *M* sodium carbonate. Put the samples, at the same time, into a boiling water bath, and continue boiling until a color change develops.

(*a*) Which of the sugars you have tried gives the test?

(*b*) Write the equation for the conversion of picric acid to picramic acid.

(*c*) Why can this test be made the basis for a colorimetric determination of sugar in the blood?

Experiment 4. Benedict Test. Add 8 drops of each of the carbohydrate solutions to 5 ml. of Benedict's qualitative reagent, and shake each tube. Place all the tubes at the same time into a boiling water bath and continue boiling for three minutes. Allow to cool and compare.

(*a*) What is the color of the precipitate?

(*b*) Discuss the chemistry of the test.

* R. Dreywood: Ind. and Eng. Chem., Analytical Ed., **18**, 499 (1946).

(*c*) What compounds other than those of copper may be used?
(*d*) What is the function of the sodium citrate?
(*e*) How does Benedict's reagent differ from Fehling's reagent?
(*f*) What substance in the urine interferes with the Fehling test?

Experiment 5. Barfoed Test. Add 1 ml. of each of the carbohydrate solutions to 3 ml. of freshly prepared Barfoed reagent. Place the test tubes at the same time into a boiling water bath and boil for one minute or longer, if necessary, until a reduction is noticed.

(*a*) Which of the sugars are oxidized?
(*b*) What is the objection to boiling the solution for too long a time?
(*c*) How does Barfoed's reagent differ from Benedict's reagent?
(*d*) Can the Barfoed test be used in place of the Benedict test for the detection of sugar in the urine?

Experiment 6. Seliwanoff Test. Add 3 drops of each of the carbohydrate solutions to 3 ml. of the Seliwanoff reagent. Place the tubes at the same time into a boiling water bath, and heat until a color develops in one or more of the solutions.

(*a*) Which solution gives the test in the shortest time?
(*b*) Could this test be used to distinguish sucrose from fructose?
(*c*) When glucose or maltose solutions containing the Seliwanoff reagent are heated for a long time, a color will also be produced. What is the reason for this?

Experiment 7. Galactose. Mucic Acid Test. Place about 50 mg. of galactose in a test tube and 50 mg. of glucose in another tube. Add 1 ml. of distilled water and 1 ml. of concentrated nitric acid to each. Heat in a boiling water bath in the hood for one and one-half hours. Add 5 ml. of water to each of the tubes, and allow them to stand overnight.

(*a*) Write the equations for the two reactions.
(*b*) What other sugar gives the mucic acid test?

Experiment 8. Phenylhydrazine Test. Using a small graduated cylinder, add 5 ml. of a freshly prepared phenylhydrazine solution to a test tube containing 2 ml. of glucose solution and to another tube containing 2 ml. of fructose solution. Place the tubes in a boiling water bath for thirty minutes. Allow the tubes to cool slowly. Remove a small quantity of crystals with a pipette, transfer them to a slide, cover with a slip, and examine under both powers of a microscope. Make drawings of the crystals.

(*a*) How do the crystalline forms of the two sugars compare?
(*b*) Write the equations for the formation of the osazones of glucose and fructose.
(*c*) Which sugars do not give osazones?
(*d*) What is the object of adding sodium acetate to the phenylhydrazine hydrochloride?

(*e*) Why is it difficult to use the melting points of osazones as a mea of distinguishing the sugars?

Experiment 9. Inversion of Sucrose. Heat 10 ml. of 1 per cent sucro solution and 2 drops of concentrated sulfuric acid in a test tube for thr minutes. Cool. Neutralize the hydrolysate with saturated sodium carbona solution, using litmus as the indicator, and perform the Benedict test.

(*a*) Does sucrose give a positive Benedict test?

(*b*) How would the hydrolysate behave towards phenylhydrazine mixtur Barfoed's and Seliwanoff's reagents?

(*c*) Explain the reactions under (*b*).

(*d*) Write the equation for the inversion of sucrose.

(*e*) What methods other than the one above can be used for hydrolyzin sucrose?

Experiment 10. Starch-Iodine Test. Add 3 ml. of a 1 per cent starc solution to each of three test tubes. To the first tube add 2 drops of water, t the second add 2 drops of 6 *N* hydrochloric acid, and to the third add 2 drop of 6 *N* sodium hydroxide. Mix. To each tube add one drop of a 0.01 *M* iodine solution. Note the color. Heat a tube in which color develops. Cool Note any change.

(*a*) What substances other than starch give a color with iodine?

(*b*) $3I_2 + 6NaOH \rightarrow 5NaI + NaIO_3 + 3H_2O$

$5NaI + NaIO_3 + 6HCl \rightarrow 3I_2 + 6NaCl + 3H_2O$

What do these equations suggest with regard to the best conditions fo performing the test?

(*c*) How would you compare the sensitivity of this test with that of the anthrone test?

Experiment 11. Hydrolysis of Starch. Mix 10 ml. of a 1 per cent starch solution with 3 ml. of 3 *M* hydrochloric acid, and immerse the tube in a boiling water bath. At three minute intervals remove a drop and test (using a test tablet) with 0.01 *M* iodine, and compare with a control. Continue the testing (observing all the color changes) until the point where the iodine color remains unchanged upon the addition of a drop of the hydrolysate. Neutralize a portion of the solution after hydrolysis with sodium carbonate, and perform the Benedict test.

(*a*) What intermediate substances are formed during the starch hydrolysis?

(*b*) How do these intermediate substances behave towards iodine and Benedict's reagent?

Experiment 12. Preparation of Potato Starch. Wash and peel potatoes. Dice 250 gm. of potato and homogenize for 30 seconds in a Waring blender with 200 ml. water. Strain the mixture through several layers of cheese cloth into a 500 ml. beaker. What is the residue? Discard.

Add 200 ml. water; stir; allow to stand undisturbed until the starch has

settled. Decant the supernatant and suspend the starch in 200 ml. water. Decant after it has settled. Suspend the starch in 100 ml. 95 per cent ethanol. Decant. Add fresh alcohol and filter on a Buchner funnel. Spread on a watch glass to dry.

Experiment 13. Time-Course of Starch Hydrolysis.

(*a*) Prepare 4 test tubes labeled 1 through 4, each with a stopper. Into each, pipette 1.0 ml. of 0.25 *M* Na_2CO_3 and 8.0 ml. water.

(*b*) Prepare a boiling water bath in a large beaker.

(*c*) Suspend 1.0 gm. of potato starch in 100 ml. of 0.5 *N* HCl in an Erlenmeyer flask fitted with a loose stopper. Shake vigorously and immediately place the flask into the water bath. Note the exact time.

(*d*) At exactly 10 minutes, remove 1 ml. of the hydrolysate by pipette and add to test tube #1. Mix.

(*e*) Repeat at 20, 30 and 60 minutes.

(*f*) Prepare 7 Folin-Wu tubes, labeled St_1, St_2, St_3, 1, 2, 3 and 4.

(*g*) Pipette into each of the appropriate Folin tubes, 2.0 ml. of each of the neutralized hydrolysates.

(*h*) Into Folin tube St_1 place 2.0 ml. of the glucose standard containing 0.1 mg. glucose per ml.; into St_2 place 2.0 ml. of the glucose standard containing 0.2 mg. glucose per ml.; into St_3 place 2.0 ml. of the glucose standard containing 0.4 mg. glucose per ml.

(*i*) To each Folin tube add 2 ml. of the alkaline copper tartrate reagent. Mix by lateral shaking. Place the tubes in a boiling water bath for 6 minutes. Cool in a beaker of cold water.

(*j*) To each Folin tube add 2 ml. phosphomolybdic acid reagent. Mix. When the evolution of CO_2 has ceased add water to the 25 ml. mark, stopper and invert several times. Read each solution against that standard which most closely matches its color intensity.

(*k*) Calculation: (Visual colorimeter; see p. 67).

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{mg. glucose in 2 ml. standard}$$
$$\times \frac{10}{2} \times 100 = \text{mg. glucose per 100 ml. hydrolysate}$$

(*l*) Plot mg. glucose per 100 ml. hydrolysate for each time interval.

(*m*) To each of the neutralized hydrolysates add 1 drop of 1 *N* HCl and 1 drop of iodine solution. Observe the color as compared with that obtained with an unhydrolyzed starch solution.

Experiment 14. Isolation of Glucosazone from a Starch Hydrolysate. Transfer 1 gm. of starch with the aid of 100 ml. of 1 *M* hydrochloric acid to a 250 ml. flask, attach a reflux condenser to the flask, and boil the contents gently to hydrolyze the starch.

At five minute intervals remove a drop of the liquid and test with iodine (Experiment 10). When the blue (starch-iodine) color no longer appears, do the Benedict test (Experiment 4) on a neutralized sample of the liquid.

Continue boiling gently for thirty minutes after a positive Benedict te has been obtained.

Transfer the hydrolysate to a beaker and, while stirring carefully, add soli sodium carbonate until the hydrolysate is neutral to litmus.

Do several carbohydrate tests on small portions of the neutralize hydrolysate (see Experiments 3, 5, and 6).

To prepare the glucosazone, add 90 ml. of phenylhydrazine mixture t the neutralized hydrolysate and boil gently over a free flame for thirty mi utes. (Caution! phenylhydrazine is poisonous and should be handled wit care. Avoid spilling the reagent or getting it on the skin. In case of such a accident, wash off with dilute acetic acid, followed by water.)

Recrystallize the osazone by dissolving it in a minimum quantity of ho 50 per cent alcohol. Add decolorizing carbon or norite, filter while hot, an cool. Examine the crystals under the microscope. Air-dry and submit th sample to the instructor.

Experiment 15. Glycogen. Grind several fresh oysters with sand in a mortar. Transfer to an evaporating dish, add 10 ml. of water, slightly acidify with acetic acid, and boil gently for fifteen minutes. **(Do not char or evaporate to dryness!)** If necessary, add 1 or 2 ml. of water. Filter. Test 2 ml. of the filtrate with 0.01 *M* iodine. In order to test the glycogen with the dilute iodine solution, use a control solution containing 2 ml. of water. Add iodine solution, drop by drop, and in the same amounts, to each. Note the development of color.

Hydrolyze 5 ml. of glycogen solution. For the hydrolysis and subsequent testing, repeat as under Experiment 14.

(*a*) Compare the iodine-glycogen test with the iodine-starch test.

(*b*) Compare glycogen and starch as to their reaction with (1) phenylhydrazine; (2) Benedict's solution, and (3) anthrone.

(*c*) Aside from the use of acid, what other methods can be used for hydrolyzing starch or glycogen?

Experiment 16. The Identification of Sucrose in the Presence of Glucose. To a mixture of 2 ml. of 1 per cent sucrose and 2 ml. of 1 per cent glucose, add 6 ml. of Benedict's qualitative reagent and immerse in a boiling water bath for ten minutes. Cool. Filter. Add 6 ml. of Benedict's solution to the filtrate and immerse in a boiling water bath again. Cool. If no reduction takes place, what does this show?

If reduction does occur, filter and treat the filtrate with Benedict's solution as before. Continue the process until no more reduction occurs. Add concentrated hydrochloric acid, a little at a time, to the filtrate until it is neutral. Now add 2 drops of concentrated hydrochloric acid and boil for three minutes. Cool. Neutralize with 0.1 *M* sodium hydroxide. Add 6 ml. of Benedict's solution and immerse in a boiling water bath.

(*a*) What is the result?

(*b*) Explain what has happened.

(*c*) What sugars are obtained when sucrose is hydrolyzed?
(*d*) How could you prove your answer under (*b*)?

Experiment 17. Identification of an Unknown Sugar. Devise your own procedure to identify the carbohydrate your instructor gave you as an "unknown."

POLARIMETRY

Ordinary light is a wave motion in which the vibrations take place in all planes perpendicular to the direction of propagation. Light caused to pass through certain substances emerges changed in such a manner that it vibrates in but a single plane, and is said to be *plane polarized*. Calcite (crystalline calcium carbonate) is frequently used to polarize light. However, the crystal causes the light to be polarized in two planes perpendicular to each other. Two wedge-shaped pieces of calcite cemented together with Canada balsam constitute a Nicol prism. Such a prism will remove one of the polarized rays and not the other (Fig. 1). The light on entering the prism is divided into

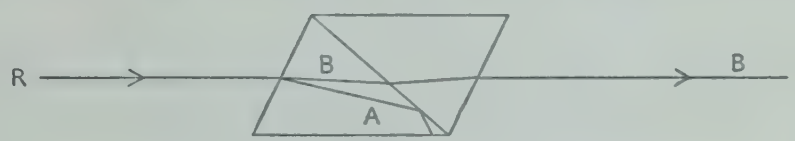


Fig. 1.

two rays, *A* and *B*. Ray *A* is bent more than *B* and is reflected from the cemented surface and absorbed in the black varnish coating the prism. Ray *B* emerges from the prism plane, polarized.

Many substances in solution have the power to rotate the plane of polarized light. Such substances are said to be *optically active*. If the rotation is measured under standardized conditions, the specific rotation is given by

$$[\alpha]_D^t = \frac{100\,\lambda}{lc}$$

where

α = specific rotation.
t = the temperature at which the measurement is made
D = the line for sodium vapor (5890 Å)
λ = observed angle of rotation
l = length of the tube in decimeters
c = the number of grams of material dissolved in 100 ml. of solution

If the specific rotation of a substance is known, the concentration of the sugar solution of unknown concentration is determined by the following relationship:

$$c = \frac{100\,\lambda}{[\alpha]_D^t\, l}$$

The percentage of substance in solution (parts by weight in 100 parts weight of solution) may be obtained if the density (d) of the solution known, by substituting $p \times d = c$ and solving for p.

$$\text{Therefore, } p = \frac{100\,\lambda}{[\alpha]_D^t \times l \times d}$$

The instrument used to determine the extent of rotation is the *polarimet* A special form designed for the examination of sugar solutions is called *saccharimeter*. These instruments vary in design, but in principle are mu the same. Monochromatic light (usually sodium D line) from the source, (Fig. 2), is passed through the lens, *B*, which renders the rays parallel. then passes through a Nicol prism, *P*, called a polarizer. The thin qua

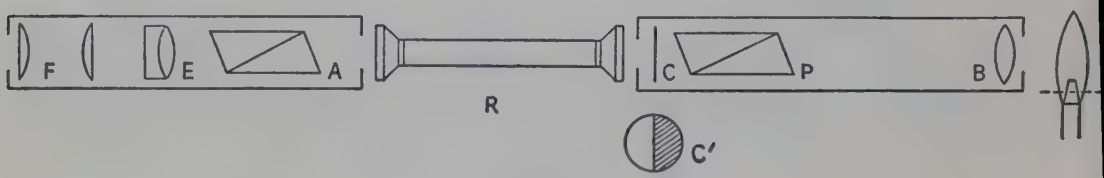


Fig. 2.

plate, *C*, half covers the field and is of such thickness as to alter the phase the light by half a wavelength. The light still remains plane polarized, b half the field is shadowed. The light then traverses the column of liquid to examined in the tube, *R*, and thence through the analyzer, *A*. The field view is observed through the telescope, *E F*. Near the eyepiece is a recordi disc which registers in fractions of a degree the rotation of the analyzer wh the two light fields are of equal intensity. (See also Fig. 3.)

Fig. 3.

Substances which rotate the beam of polarized light to the right are called *dextrorotatory*, and those rotating it to the left, *levorotatory* (not to be confused with the D- and L- forms, which indicate chemical structural relationships and not rotations).

Some freshly prepared sugar solutions change their rotation with time. This is called *mutarotation*. When measuring the rotation of such substances, it is important to make certain that equilibrium among the various forms has taken place.

Before using the instrument, consult the specific directions which come with the polarimeter or saccharimeter, or consult your instructor.

Calculation of Specific Rotation: Twenty grams of sucrose are weighed and dissolved in water to 100 ml. This solution gives an angular rotation for sodium light of $+53.2°$ in a 400 mm. tube at 20°C.

Calculate the specific rotation of sucrose for the given concentration.

Experiment 18. Determination of Specific Rotation. Rinse a polarimeter tube twice with 5 to 10 ml. portions of distilled water, and then fill to the top with water, and stopper. Place in the polarimeter and rotate the analyzer of the polarimeter until both halves of the circular field seen through the eyepiece are equally illuminated. Change the analyzer setting and again bring the analyzer to the point where the field is uniform and obtain another reading. In this way take three readings and calculate the average. This is the zero reading of the polarimeter.

Dissolve 2 gm. of D-glucose in about 95 ml. of distilled water in a 100 ml. volumetric flask. Add several drops of concentrated ammonium hydroxide, or some solid sodium carbonate, until 1 drop of the sugar solution gives a red color to 2 drops of phenolphthalein solution (which is used as an outside indicator). Now make up to 100 ml. with distilled water. Mix well.

Rinse the polarimeter tube at least twice with 5 to 10 ml. of the clear, colorless sugar solution, fill with that solution, and take at least three readings in the polarimeter as before. Calculate the average of these readings. Calculate the difference between the zero reading and the reading of the optically active solution. Record the temperature.

Repeat this procedure with sucrose and maltose. Calculate the specific rotation of each sugar and compare with those given in the literature.

(*a*) Why is it necessary to add ammonium hydroxide or sodium carbonate to each reducing sugar before reading in the polarimeter?

(*b*) Explain the terms "dextrorotatory" and "levorotatory." Name one carbohydrate used in this experiment which is dextrorotatory and one which is levorotatory.

(*c*) What is the meaning of the terms D-glucose and D-fructose?

(*d*) Write the formulas of the compounds believed to be present in an equilibrium mixture of glucose and of fructose.

(*e*) List some reasons for the difference between your experimentally determined values of specific rotation and those in the table.

Experiment 19. Rate of Acid Hydrolysis of Cane Sugar. Turn on the sodium lamp about 20 to 30 minutes before taking polarimeter readings.

Heat a water bath to 60°C. and regulate the burner so that the water ba remains approximately constant at this temperature. Obtain the zero readi of the polarimeter as in the previous experiment.

Prepare 200 ml. of a solution containing 20 gm. of sucrose and mix we Place the sugar solution in the water bath and allow to remain in the wat bath for 5 minutes. Add 10 ml. of 6 *M* hydrochloric acid, mix well, a immediately remove by pipette 10 ml. of the hydrolysis mixture. Determi the minimum number of drops of 2 M sodium hydroxide solution required impart a pink color to phenolphthalein, which is used as an outside indicat (2 to 3 drops usually suffice). At five minute intervals remove 10 ml. aliquo and add the same quantity of sodium hydroxide solution to each. Contin until eight samples have been collected. Allow each to come to room temper ture and obtain the polarimeter reading for each aliquot.

Plot the results and calculate the rate of hydrolysis. Also plot on sem log paper.

(*a*) Why is the hydrolysis of sucrose called inversion?

(*b*) Why is sodium hydroxide added to the mixture before reading in th polarimeter?

(*c*) Devise another method to carry out this experiment.

(*d*) How and where is sucrose hydrolyzed in the body?

Experiment 20. Determine polarimetrically the percentage of the car bohydrate in the solution given you by your instructor.

Chapter 2 Lipids

THE LIPIDS may be separated from one another by taking advantage of their solubilities in various organic solvents. With the exception of the uncombined sterols, all lipids may be hydrolyzed by alkalies, yielding soaps which are insoluble in ether; so that the fatty acids may be removed from the other hydrolytic products.

Experiment 1. Bisect an egg through its long axis by a sharp blow of a metal spatula. Holding the egg over a 400 ml. beaker, separate the two halves of the shell, roll the contents of the egg from one shell to the other, permitting as much as possible of the egg white to slip down into the beaker.

Save the egg white, add one drop of toluene and place it in the refrigerator for use under proteins (Chap. 3). Stir the yolk with a solution of 50 ml. of alcohol and 25 ml. of ether. Allow to stand for ten minutes, stirring at intervals. Filter through a filter paper moistened with alcohol into a dry beaker. Wash the residue in the filter paper with a fresh 20 ml. portion of the alcohol-ether solution used for the extraction. (Beware of flames!)

Evaporate the filtrate to dryness on a steam bath. Dissolve the residue in 10 ml. of ether. Slowly pour the solution into 30 ml. of acetone, stirring during the addition. The precipitate is mainly lecithin. Filter.

Without too much delay, convert the lecithin to the cadmium chloride addition product (Experiment 2). (If lecithin remains exposed to the air for any length of time, it darkens.)

Evaporate the ether-acetone filtrate (from the lecithin precipitation) to a paste on the steam bath. Cool. Add 15 ml. of 10 per cent alcohol potassium hydroxide solution. Stir well and heat the solution for thirty minutes on the steam bath. Cool. Add 50 ml. of ether. Filter the precipitate of soap (reserve for tests under Experiments 6 to 8). The ethereal filtrate contains cholesterol. Evaporate to dryness on the steam bath.

Extract the cholesterol by warming the residue with 5 ml. of alcohol on the steam bath. Transfer the warm alcoholic solution to a dry 15 ml. centrifuge tube with the aid of a medicine dropper. Repeat the extraction with 3 ml. more of the alcohol. Centrifuge the alcoholic solution for two or three minutes to precipitate all insoluble debris. (The instructor will demonstrate the proper use of the centrifuge.) Transfer the supernatant alcoholic solution to another clean dry centrifuge tube with the aid of a dry, long medicine dropper. Add water to the solution, dropwise, until no more precipitate is obtained (about 5 to 10 drops). Let stand for a half hour. Centrifuge. Decant the clear supernatant. The crystalline residue in the centrifuge tube can be recrystallized in the tube by dissolving in the minimum of hot alcohol and allowing to cool. When cool, a few drops of water will complete the crystallization. Centrifuge again and after decanting the supernatant, dry the crystals in a vacuum desiccator. About 50 to 75 mg. of cholesterol can be obtained from

one yolk by this method. Examine the crystals under a microscope and sket a few of them. Perform the Liebermann-Burchard test on a few cryst (Experiment 10).

(*a*) Write the formula of free and a combined cholesterol.
(*b*) How would you distinguish these two forms of cholesterol?
(*c*) Why is alcoholic instead of aqueous potassium hydroxide used f the saponification?

Experiment 2. Phospholipids. Lecithins and cephalins (as well other phospholipids) yield a crystalline addition product when treated wi alcoholic cadmium chloride. Upon hydrolysis of lecithin or cephalin, fat acids, glycerol, phosphoric acid and a nitrogenous base are obtained.

Dissolve the phospholipids (see Experiment 1) in 20 ml. of alcohol. Ad an equal volume of alcoholic cadmium chloride solution. Stir and allow t stand for ten minutes. Filter on a Buchner funnel and dry the precipitate.

Test small portions of the dry addition product for glycerol (see Exper ment 3), and phosphorus (see Experiment 12). Place the remainder in clean, dry vial; label it and submit it to your instructor.

(*a*) Lecithin is used as an edible emulsifying agent in chocolate bars Why is it so effective for this purpose?
(*b*) What is the source of commercial lecithin?
(*c*) What happens to lecithin on exposure?

Experiment 3. Acrolein Test (for Glycerol). Rub in a mortar abou 0.1 gm. of dry material to be tested with an equal quantity of potassium bisulfate. Transfer to a dry test tube. Heat gently at first and then mor strongly. Note the odor.

(*a*) Formulate this reaction.

Experiment 4. Foam Test (for Soaps). Add a few ml. of water to small sample to be tested and shake vigorously. A foam or suds, which should remain for several minutes, forms.

(*a*) What is a foam?
(*b*) Why does it form?

Experiment 5. Saponification Test (for Combined Fatty Acids). Add 10 ml. of 10 per cent alcoholic potassium hydroxide to the sample to be tested, stir and heat on a steam bath until a drop of the solution is completely soluble in water. Now add 10 ml. of water and heat on the steam bath until all the alcohol has been driven off. Perform the foam test.

(*a*) What substances other than soaps produce a foam?

Experiment 6. Precipitation Test (for Soaps). Add concentrated hydrochloric acid dropwise to a cold aqueous solution of the substance to be

ested until the mixture is strongly acid to Congo red paper. Shake well during the addition of the acid. Result?

(*a*) What other organic compounds give this reaction?

Experiment 7. Salting Out (for Soaps). To 5 ml. of the soap solution dd a little solid sodium chloride. Stir until the salt dissolves. Repeat this ntil no more sodium chloride dissolves. What happens to the soap?

(*a*) What is an industrial application of this experiment?

Experiment 8. Insoluble Soaps. To 5 ml. of the soap solution add 2 ml. of 0.5 *M* calcium chloride. Result? To another 5 ml. portion of the soap solution add 2 ml. of 0.5 *M* lead nitrate. Result?

(*a*) What is hard water?

Experiment 9. Salkowski Test for Cholesterol. (Neither this test nor the Liebermann-Burchard test [Exper. 10] can be performed successfully unless the glass vessels, reagents and the substance being tested are dry.) Dissolve a few milligrams of the substance to be tested in 3 ml. of anhydrous chloroform in a dry test tube. Add an equal volume of concentrated sulfuric acid (specific gravity, 1.84) and shake the tube gently. Let the liquid layers separate, and note the colors.

(*a*) Which is the chloroform layer? What is its color?

(*b*) Describe the color of the sulfuric acid layer in reflected light and in transmitted light.

Experiment 10. Liebermann-Burchard Test for Cholesterol. Prepare a chloroform solution of the substance to be tested as in Experiment 9. Add 10 drops of acetic anhydride and 2 drops of concentrated sulfuric acid. Shake gently and allow to stand for a few minutes.

(*a*) Describe the color of the contents of the tube.

(*b*) How would you compare the sensitivity of these two tests (Experiments 9 and 10) for cholesterol?

(*c*) What features of this reaction make it useful for quantitative measurement?

Experiment 11. Digitonin Precipitation. Dissolve 1 to 2 mg. of cholesterol in 2 ml. of alcohol. Add to this solution 2 ml. 1 per cent digitonin in 80 per cent alcohol-water solution.

(*a*) What is the precipitate?

(*b*) What compounds other than cholesterol give this reaction?

(*c*) Would a cholesterol ester be precipitated by digitonin?

Experiment 12. Test for Phosphorus.* *A.* FUSION. (It is advisable to wear goggles during this experiment.) Support a clean, dry, 4 by ½ inch

* From "A Guide to Qualitative Organic Analysis," by Davidson and Perlman (Brooklyn College Press).

test tube of soft glass in an asbestos square which rests on an iron ri Introduce a 3 mm. cube of sodium and heat the tube gently until a dark clo of sodium vapor rises about 1 cm. Remove the flame momentarily and imm diately drop about 10 mg. of the dry substance to be tested to the botto of the test tube. Heat the tube and its contents a little more strongly a repeat the removal of the flame and the addition of the substance twice mor Finally heat strongly to complete the decomposition, remove the burner a immediately raise a 50 ml. beaker containing 10 ml. of water until the wat is in contact with the hot tube momentarily. After the first violent reactio repeat the contact and finally break off the bottom of the test tube by strikin it against the bottom of the beaker. (During this operation the mouth of th test tube should point upward and the beaker should be held right up again the asbestos to protect the experimenter from flying glass or sodium.)

Macerate thoroughly with a stirring rod, filter through a fluted filter int a large test tube, cool the filtrate and use it for the following test. (A success ful fusion is indicated by the presence of flakes of carbon in the filter pape and a clear, colorless filtrate.)

B. PHOSPHORUS TEST. The odor of phosphine during the preparation o the stock solution indicates the presence of phosphorus. To confirm it, ad into a 6 by ¾ inch test tube 1 ml. of the solution and 2 ml. of concentrate nitric acid. Boil the solution down to one-half of its volume under a hood Add 3 ml. of ammonium molybdate solution and warm the tube to 60°C Result?

(*a*) What are the compounds produced by the sodium fusion of a com pound containing nitrogen and phosphorus?

(*b*) What lipids contain nitrogen as well as phosphorus?

ANALYSIS OF FAT

Experiment 13. Saponification Number.* This is the number o milligrams of potassium hydroxide required to saponify 1 gm. of fat. Using equal weights, a fat of low molecular weight will require more potassium hydroxide to saponify it than a fat of high molecular weight.

PROCEDURE. Weigh 5 gm. of the fat into a 250 to 300 ml. Erlenmeyer flask. Measure out by means of a burette 50 ml. of the alcoholic potassium hydroxide solution into the flask.

Connect the flask with an air condenser and boil until the fat is completely saponified (about thirty minutes). Cool and titrate with 0.5 *N* hydrochloric acid, using phenolphthalein as indicator. (For the preparation of standard hydrochloric acid, see page 147.)

Perform a blank determination.

Subtract the number of milliliters of 0.5 *N* hydrochloric acid obtained in the determination of the sample from the number obtained in the blank to obtain the number of milliliters of 0.5 *N* hydrochloric acid equivalent to the potassium hydroxide used in the saponification of the sample.

* Methods of Analysis, A.O.A.C. (1935), p. 412; Jacobs, Chemical Analysis of Foods and Food Products (1938), p. 218.

Calculate as saponification number the milligrams of potassium hydroxide required to saponify 1 gm. of fat.

REAGENTS:

ALCOHOLIC POTASSIUM HYDROXIDE SOLUTION. Reflux 1.2 liters of 95 per cent alcohol for thirty minutes in a 3 liter flask with 10 gm. of potassium hydroxide and 6 gm. of aluminum foil. Distil and collect 1 liter, discarding the first 50 ml. Dissolve 40 gm. of potassium hydroxide in this liter of alcohol. Keep the solution in a stoppered bottle in the dark.

The following data were obtained during the determination of the saponification number of a sample of beef tallow:

Weight of change, 5.25 gm.
Volume of potassium hydroxide solution, 50 ml.
Volume of 0.52 *N* hydrochloric acid to titrate excess potassium hydroxide, 40 ml.
Volume of 0.52 *N* hydrochloric acid to titrate blank, 95 ml.

Calculate the saponification number.

Experiment 14. Iodine Number.* Fats contain varying amounts of unsaturated fatty acids which absorb iodine. The amount of iodine absorbed is a measure of the extent of unsaturation of the fat. The result—the "iodine number"—is expressed as the number of grams of iodine absorbed by 100 gm. of fat.

Two general methods are used: the Hanus method, which uses iodine bromide as a carrier, and the Wijs method, which uses iodine chloride. The Hanus method will be described.

PROCEDURE. Weigh 0.25† gm. of fat into a 500 ml. glass-stoppered flask. Dissolve the sample in 10 ml. of chloroform. Add 30 ml. of Hanus iodine solution and let stand thirty minutes with occasional shaking.

Add 10 ml. of 15 per cent potassium iodide solution, shake thoroughly, and add 100 ml. of freshly boiled and cooled water, washing down any free iodine that may be found on the stopper. Titrate the iodine with 0.1 *N* sodium thiosulfate until the yellow color of the solution has almost disappeared. Add 2 ml. of a 1 per cent starch solution (as indicator) and continue the titration until the blue color has disappeared.

Towards the end of the titration, stopper the bottle and shake vigorously, so that any iodine remaining in solution in the chloroform will be taken up by the potassium iodide solution. Complete the titration.

Run two blank determinations on an equal portion of the Hanus solution.

The number of milliliters of the 0.1 *N* sodium thiosulfate solution required by the blank, less the quantity used in the determination, gives the thiosulfate equivalent of the iodine absorbed by the fat. Calculate the grams of iodine absorbed by 100 gm. of the fat. This is the Hanus iodine number.

REAGENTS:

HANUS IODINE SOLUTION. Dissolve 13.2 gm. of pure iodine in 1 liter of

* Methods of Analysis, A.O.A.C. (1935), p. 410; Jacobs, Chemical Analysis of Foods and Food Products (1938), p. 213.

† Actually, 0.15 gm. to 1.0 gm., depending on the oil or fat: 0.15 for most oils, 0.25 for lard, 0.3 for beef or mutton tallow, 0.4 for butterfat, 1.0 for cocoanut oil.

glacial acetic acid (99.5 per cent). Add enough bromine to double th halogen content (about 3 ml.).

0.1 *N* Sodium Thiosulfate Solution. Prepare a solution containin 24.82 gm. of sodium thiosulfate ($Na_2S_2O_3.5H_2O$) in freshly boiled and cool water and dilute to 1 liter. Standardize this solution as follows: Place in glass-stoppered flask 20 ml. of the 0.1 *N* potassium dichromate, and 10 ml. the 15 per cent potassium iodide solution. Add 5 ml. of hydrochloric aci Dilute with 100 ml. of freshly boiled and cooled water and allow the 0.1 sodium thiosulfate to flow slowly into the flask until the yellow color of th liquid has almost disappeared. Add a few drops of starch indicator and, wit constant shaking, continue to add 0.1 *N* sodium thiosulfate until the blue colo just disappears.

0.1 *N* Potassium Dichromate. Dissolve 4.903 gm. of potassiu dichromate in water and dilute to 1 liter.

(*a*) The following data were obtained during the determination of th iodine number of a sample of beef tallow:

Weight of charge, 0.31 gm.
Volume of 0.1 *N* thiosulfate needed to titrate blank determination, 62.5 ml.
Volume of 0.1 *N* thiosulfate needed to titrate determination, 48 ml.

What is the iodine number of the tallow?

Experiment 15. Chromatographic Analysis.* Chromatographic anal ysis offers an excellent technic for the separation of mixtures of biological compounds closely related in chemical structure.

The separation depends on the fact that compounds with but slight differ ences in structure will be selectively adsorbed to different degrees when various adsorbents are used.

It is of interest to note that the natural vitamin D, which occurs in fish liver oils, has been isolated by this method, the compound having been first converted into its 3,5-dinitrobenzoate, so that its yellow color could be used as a visual aid in following its course down a column of activated aluminum oxide.

Procedure. Immerse 3 or 4 fresh, crushed spinach leaves in a mixture of 45 ml. of petroleum ether (B.P. 70°C.), 5 ml. of benzene and 15 ml. of methanol.

Allow to stand for one hour. Remove the residue, filtering by suction, and wash with some of the solvent mixture.

Transfer the extract to a separatory funnel and wash out the methanol with water without shaking vigorously. Dry the extract over anhydrous sodium sulfate.

For the adsorption, use a tube 18 cm. long and 1 cm. in diameter having a constriction near the lower end (Fig. 4).†

* Tswett, *Ber.*, *24*, 234, 361, 384 (1906); Winterstein and Stein, *Z. Physiol. Chem.*, *220*, 247 (1933); Cook, *J. Soc. Chem. Ind.*, *55*, 724 (1936); Gatterman and Wieland, *Laboratory Methods of Organic Chemistry* (1937), p. 410.

† Columns fitted with a ground glass plate and interchangeable joints are excellent for this purpose.

Fit a cotton plug into the constricted portion of the tube using a glass rod hat just fits the inner bore of the tube.

Pour aluminum oxide into the tube, small portions at a time, tamping down ith the glass rod until a layer 2 cm. in depth is formed.

In the same way form a layer of calcium carbonate (dried at 150°C.) cm. high.

Cover with a layer of finely powdered sugar ("icing sugar") 6 cm. in ngth.

Cover the sugar layer with some cotton.

Run in petroleum ether (B.P. 70°C.) from a dropping funnel, maintaining

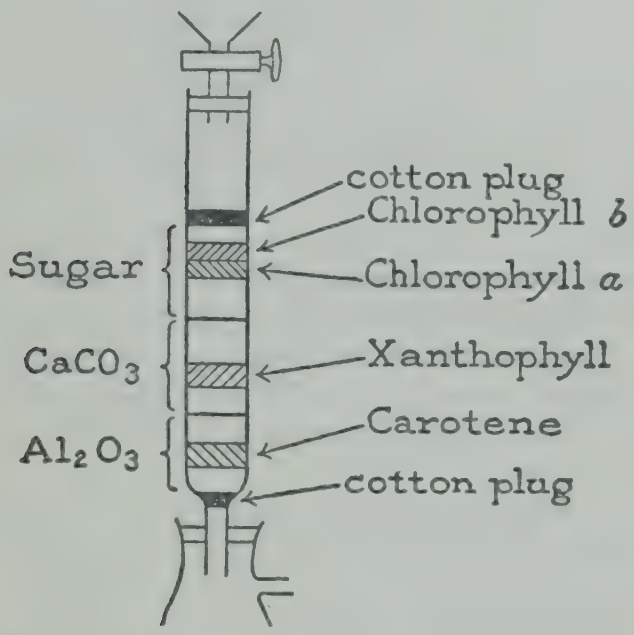


Fig. 4.—Apparatus for chromatographic analysis.

a gentle suction* so that the filtrate comes through at the rate of 1 to 2 drops per second.

Follow the petroleum ether with the green extract.

When all of the extract has been added, develop the colored zones by adding a 4:1 mixture of petroleum ether and benzene.

Finally wash with petroleum ether (B.P. 30°–50°C.) to check any extension of the zones.

Suck the column dry in a stream of carbon dioxide or nitrogen gas.

Cut the column between the colored zones and dissolve the pigments with ethyl ether containing a little methanol.

If a spectroscope is available, examine the absorption spectrum of each colored extract.

How could this method be used to separate colorless mixtures?

* It is important to keep the adsorbent constantly covered with solvent to at least 1 cm. above the top layer.

Chapter 3 **Proteins**

PREPARE a dilute egg albumin solution (1:10) from the egg white saved from the previous experiment on lipids (see p. 11) by stirring vigorously one volume of egg white with five volumes of water. Filter through a fluted filter paper and use this solution for the tests which follow.

IDENTIFICATION TESTS

A number of qualitative color reactions have been devised which are useful for the detection of proteins. Owing to the complexity of the protein molecule and the difficulty of obtaining a single, pure protein compound, these tests are to be used with the knowledge that they test for specific chemical groupings in the protein molecule, or more generally for groupings which most proteins contain. Thus Millon's reagent gives a characteristic color with the hydroxyphenyl group; and a positive reaction is given only by those proteins which contain tyrosine. Ninhydrin, however, gives a positive test with any substance having an alpha-amino group, and a positive biuret test depends on the peptide bond. Since no one test is absolutely specific for proteins, it is necessary to apply several tests.

Perform experiments 1-5 on each of the following solutions: (*a*) diluted egg albumin solution; (*b*) gelatin solution (2 per cent); (*c*) water (control).

Experiment 1. Millon Test. Add 5 drops of Millon's reagent to 3 ml. of the protein solution. Carefully heat the mixture. (If too much reagent is used, the color may disappear on boiling.)

(*a*) What happens when a mercury salt is added to proteins?
(*b*) What constitutes a positive reaction?
(*c*) Why does the albumin solution coagulate?
(*d*) Why cannot the reaction be performed in a strongly alkaline medium?
(*e*) What type of aromatic substance gives this test?
(*f*) Which of the protein solutions gives a weak or negative test? Why?
(*g*) How would you adapt this reaction for a quantitative determination?

Experiment 2. Xanthoproteic Test. Add, slowly, 1 ml. of concentrated nitric acid to 3 ml. of the protein solution. Heat gently. Cool the solution under the tap, and add, cautiously, drop by drop, concentrated sodium hydroxide (or concentrated ammonium hydroxide). What happens when the alkali comes in contact with the liquid? Continue the addition of the sodium hydroxide until the solution is alkaline.

(*a*) What happens when a strong acid is added to a protein solution?
(*b*) What change is produced when the protein is heated with nitric acid?
(*c*) What is the effect of the added alkali?

(*d*) Why does nitric acid stain the skin a yellow color?
(*e*) What aromatic groups of the protein molecule are responsible for the test?

Experiment 3. Hopkins-Cole Test. Add 2 ml. of the reagent to 2 ml. of the protein solution. Mix. Incline the tube and add, slowly, 5 ml. of concentrated sulfuric acid down the side of the tube. Note the color formed at the junction of the two liquids. If necessary, gently rotate the tube to develop the colored ring.

(*a*) Write the formula for glyoxylic acid.
(*b*) What constituent in the protein molecule is responsible for the test?
(*c*) What protein fails to give this test? Why?

Experiment 4. Biuret Test. Add 1 ml. of concentrated sodium hydroxide to 3 ml. of the protein solution and mix. Add a drop of 0.01 *M* copper sulfate. Mix. If no decided color develops, add another drop or two of copper sulfate.

(*a*) What color is developed?
(*b*) Why is an excess of copper sulfate to be avoided?
(*c*) Why do ammonium salts interfere?
(*d*) What grouping in the protein molecule is responsible for this test?
(*e*) Name two substances other than proteins which give a positive biuret test.

Experiment 5. Ninhydrin Test. Add 0.5 ml. of a 0.1 per cent solution of triketohydrindene hydrate (ninhydrin) to 3 ml. of the protein solution. Heat to boiling.

(*a*) What color is developed?
(*b*) What is the formula for ninhydrin?
(*c*) What grouping gives a positive test?
(*d*) How could this test be adapted for the quantitative determination of amino acids?

Devise such a determination.

Experiment 6. Cystine. Dissolve a little cystine in 5 ml. of 1 *N* NaOH. Add a few crystals of lead acetate. Heat to boiling.

(*a*) What is the color of the precipitate?
(*b*) What is the precipitate?

Experiment 7. Cysteine. Dissolve a few crystals of cysteine hydrochloride in 5 ml. water. Add 0.5 ml. of a fresh solution of 1 per cent sodium nitroprusside. Add 0.5 ml. ammonium hydroxide.

(*a*) What is the color?
(*b*) What grouping in cysteine is responsible for the test?
(*c*) Is the color stable?

(*d*) Will cystine give a positive test?

(*e*) What important peptide of cysteine will give a positive nitropruss test?

Experiment 8. Conversion of Cystine to Cysteine. Dissolve so cystine in 0.5 *N* HCl in a test tube. Add a pinch of zinc dust and allow t reaction to proceed for 5 minutes, warming gently if necessary. Filter a apply the nitroprusside test as in Experiment 7.

Experiment 9. Conversion of Cysteine to Cystine. Dissolve so cysteine hydrochloride in 5 ml. water. Add a few drops of ferrous sulfa solution. Observe any color change. Add more of the ferrous sulfate sol tion until no further change takes place. Test the resulting solution wi nitroprusside (Experiment 7).

(*a*) Write the reactions which take place in 8 and 9.

(*b*) Of what significance is the ability of ferrous salts to oxidize th sulfhydryl groups of cysteine?

Experiment 10. Filter Paper Chromatography.* The separation compounds in solution by differential adsorption has a long history. Th use of filter paper instead of a column packed with adsorbent (p. 17) wa made most popular by the work of Martin, Consden, Gordon and Synge (se for example, Consden et al., Biochem. J. *38*, 224, 1944). A mixture of sub stances in solution is applied as a drop near one end of a filter paper strip The drop is dried and that end dipped into a solvent mixture. The pape strip may be hung for descending solvent flow or dipped into the solvent fo ascending solvent flow. As the solvent mixture flows by the mixture o solutes, they dissolve and are carried along by the solvent at different rate The extent of movement depends on the type of filter paper used and th nature of the solvents. The factors which affect the separation are usuall a combination of partition, adsorption and ion-exchange. The followin experiment is designed to introduce the student to the technique in it simplest form and has the advantage of being completed in a single laborator period.

(*a*) Prepare 5 strips of Whatman No. 1 filter paper having the dimen sions 13.5 $\times$ 1.80 $\times$ 1.00 cm. (Fig. 5). Test the fit of these papers in the tes tubes to be used.

(*b*) Lightly pencil a circle 1.5 mm. in diameter about 6 mm. from th narrow end of each paper. Fill a capillary pipette with a small volume of th appropriate solution so as to contain approximately 20 micrograms of th amino acid. Quickly and gently touch the end of the pipette to the cente of the circle. The wet area should not spread beyond the circle. Dry betwee applications if it is necessary to apply more. Finally dry all spots. Prepar the following:

Tube No. 1—isoleucine
Tube No. 2—threonine

* Rockland, L. B., and Dunn, M. S. Science, *109*, 539, 1949.

Tube No. 3—aspartic acid
Tube No. 4—mixture of 1, 2 and 3
Tube No. 5—unknown amino acid (one of the above)

(*c*) Pipette 0.4 ml. of water-saturated phenol into the bottom of each test tube.

(*d*) Place each paper strip, narrow end down, into its appropriate tube. Only the top portion of the paper should touch the sides of the test tube.

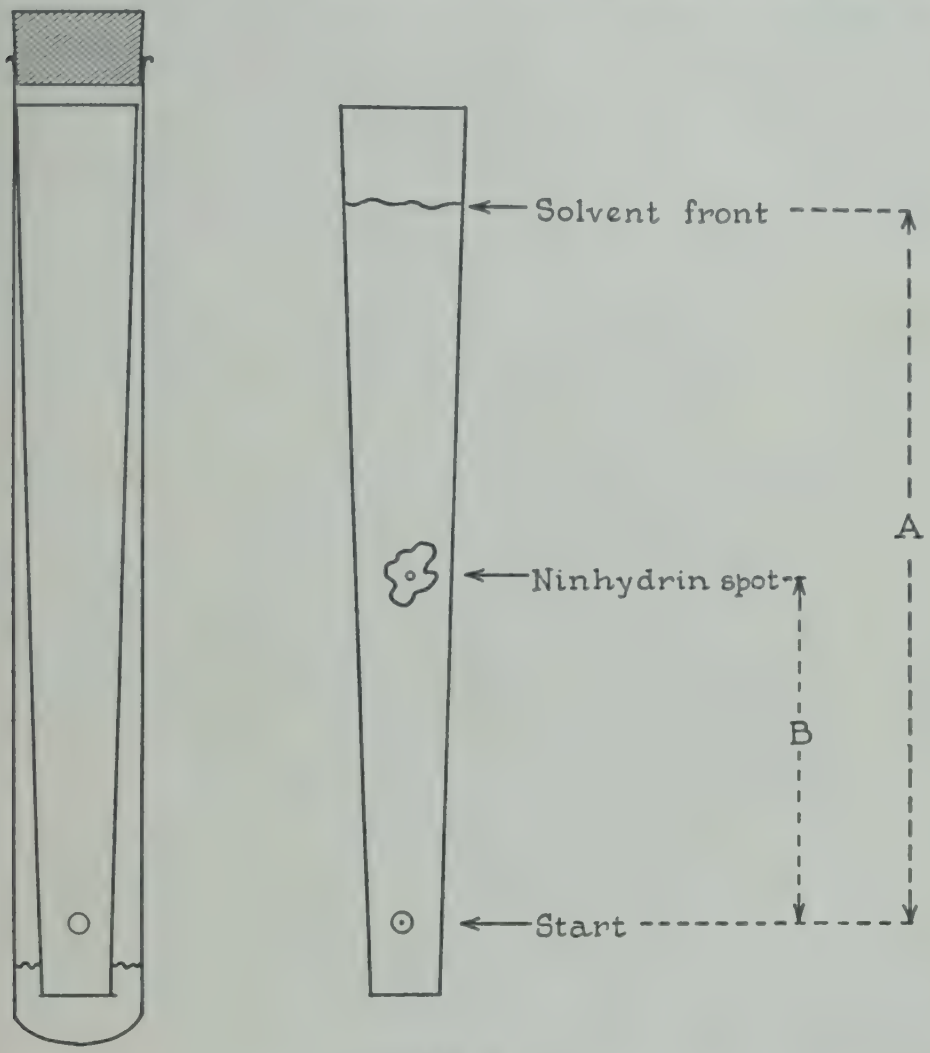


Fig. 5.

The bottom should dip into the solvent mixture. Stopper and allow to stand for 2 to 3 hours until the solvent front has traveled to about 5 mm. below the top of the paper.

(*e*) Remove each paper and mark the position of the solvent front. Dry at 110° for 3 minutes and gently spray with a 0.25 per cent solution of ninhydrin in water-saturated butanol. Dry at 110° and circle the colored spots.

(*f*) In each case measure the distance traveled by the solvent front (A) and the distance traveled by the amino acid (B). Calculate the R_f value for each amino acid; $R_f = \frac{B}{A}$

(*g*) Identify the unknown. Suggest modifications. Could this method used for a mixture of many amino acids?

Table 1

Amino acid	R_f *(3 hrs.; 125 mm. at 25°)*
Alanine	0.62
Arginine HCl	0.53
Aspartic acid	0.25
Glutamic acid	0.39
Glycine	0.49
Histidine HCl	0.81
Isoleucine	0.85
Leucine	0.86
Lysine HCl	0.41
Methionine	0.74
Phenylalanine	0.87
Proline	0.87
Serine	0.33
Threonine	0.57
Tryptophan	0.81
Tyrosine	0.53
Valine	0.82

PROPERTIES OF PROTEINS

Insoluble salts may be formed when heavy metal ions or the alkaloid reagents are added to proteins in solution. Very soluble salts may compe for water with the proteins and so precipitate them. If the solution is sat rated with ammonium sulfate, all proteins and breakdown products to th peptone stage will be precipitated. By varying the extent of saturation wi these salts, albumins may be separated from globulins.

A more recent method of precipitating and separating proteins has bee applied extensively to the fractionation of the serum proteins by E. J. Coh and his group at Harvard University. This method makes use of the fact th precipitation of a protein from solution by alcohol at very low temperatur (—5 to 0°C.) will avoid denaturation. The conditions which may be varie are (*a*) ionic strength, or concentration of electrolyte, (*b*) *p*H, (*c*) alcoh concentration and (*d*) concentration of the protein.

Experiment 11. Precipitation by Metallic Salts. To 3 ml. of the pr tein solution add 5 drops of 0.2 *M* mercuric chloride. Repeat, using 0.2 lead acetate instead of the mercury salt. Results? Explain.

Why is egg white used as an antidote for lead and mercury poisoning?

Experiment 12. Precipitation by Alkaloidal Reagents. Place 3 m of protein solution in each of three test tubes. To one add 5 drops of sat rated picric acid solution. To another add 10 per cent tannic acid solutio To the third, acidified with 0.1 *M* hydrochloric acid, add phosphotungst acid reagent. Results?

What are the alkaloidal reagents?

Experiment 13. Heller Test. Place 3 ml. of concentrated nitric acid in a test tube, incline it, and, using a pipette, add slowly down the side of the tube an equal volume of protein solution.

(*a*) Explain the result.
(*b*) What practical application does this test find?
(*c*) Compare with the xanthoproteic test.

Experiment 14. Salting Out. Saturate 10 ml. of the protein solution with ammonium sulfate. To do this, first add a small quantity of the salt, stir until it goes into solution, then add a little more ammonium sulfate, and stir again; and continue the process until a little of the salt remains undissolved. When the solution is saturated, filter. Test the solubility of the precipitate in water. Test the precipitate with Millon's reagent and the filtrate by the biuret test. (Recall the effect of ammonium salts in testing with biuret reagent.)

(*a*) Explain the result.
(*b*) What proteins are precipitated by saturating their solution with ammonium sulfate; with 50 per cent ammonium sulfate?

Experiment 15. Coagulation Test. Add 2 drops of *M* acetic acid to 5 ml. of the protein solution. Put the tube in a boiling water bath for five minutes. Collect the precipitate on a stirring rod. Test the solubility of the precipitate in water. Test some of it with Millon's reagent.

(*a*) Why is the acid added?
(*b*) What proteins coagulate on boiling?

Experiment 16. Denaturation and Coagulation. Make up the following three mixtures in test tubes:

Test Tube	*1*	*2*	*3*
Egg albumin	9 ml.	9 ml.	9 ml.
Acetate buffer, *p*H 4.7 (1 *M*)	—	—	1 ml.
HCl (0.1 *M*)	1 ml.	—	—
NaOH (0.1 *M*)	—	1 ml.	—

Place all three tubes in a boiling water bath for fifteen minutes and cool to room temperature. In which tubes do precipitates appear? To tubes (1) and (2) add 10 ml. of acetate buffer, *p*H 4.7. Note the result.

(*a*) What physical property of proteins influences the solubility of the protein in this experiment?
(*b*) What other methods may be used to denature proteins?
(*c*) What chemical change is associated with the denaturation of egg albumin?

Experiment 17. Precipitation of Proteins by Alcohol. Set up the following three test tubes:

Test Tube	*1*	*2*	*3*
Dilute egg albumin	5 ml.	5 ml.	5 ml.
0.1 *M* HCl	1 ml.	—	—
0.1 *M* NaOH	—	1 ml.	—
Acetate buffer, *p*H 4.7	—	—	1 ml.
Ethyl alcohol, 95 per cent	6 ml.	6 ml.	6 ml.

Which tubes show the presence of insoluble proteins? Is the egg albu soluble in water at its isoelectric point?

Experiment 18. Test for Nitrogen. *A.* FUSION. See p. 13 for preparation of the solution resulting from the sodium fusion.

B. NITROGEN TEST. Stir 0.25 gm. of ferrous sulfate with 0.5 ml. water in a 4 by ½ inch test tube. Add 1 ml. of the solution prepared in (*A* mix well, then add 1 ml. of 2 *M* sodium hydroxide, mix again, and then a concentrated hydrochloric acid dropwise until the precipitated ferro hydroxide redissolves. If there is nitrogen in the substance being teste prussian blue appears as a dark blue precipitate or a green suspension.

(*a*) What are the compounds produced by the sodium fusion of a co pound containing nitrogen?

Experiment 19. Test for Sulfur. Mix 0.5 gm. of dry egg albumin wi twice its weight of fusion mixture (see Appendix). Heat in a porcelain di or crucible until clear. Cool and dissolve in warm water. Filter if necessar Acidify the filtrate with HCl, heat to boiling and add a few drops of BaC solution.

(*a*) Why do proteins give a positive test for sulfur?

(*b*) In what form is phosphorus often found in proteins?

(*c*) What common elements are present in proteins which are absent fats and carbohydrates?

Experiment 20. Dialysis. See p. 52 under Milk.

Experiment 21. Donnan Membrane Equilibrium. To 2 gm. of gel tin add 100 ml. of hot water (90°C.) and stir until a homogeneous solutio is obtained. Cool to room temperature and add 5 ml. of 0.04 per cent bro phenol blue solution. Place 100 ml. of water in a 400 ml. beaker and add 2 ml. of the indicator. Add 0.01 *M* hydrochloric acid to the water, drop b drop, until the solution just turns a definite yellow. Now add a drop or tw of 0.01 *M* sodium hydroxide so that the yellow solution just changes to th light reddish-purple or pale blue color (*p*H 4.6). (The dye is dichromati reddish-purple by reflected light and blue by transmitted light.)

Using 0.1 *M* hydrochloric acid and sodium hydroxide, treat the gelati solution in the same manner, so that its color is as close as it is possible get it to the color of the water. Pour the gelatin solution into a dialyzing bag

* An excellent substitute for collodion membranes may be obtained from the Viski Corporation, Chicago, Illinois. The membrane is made wet with water, blown ope and one end knotted on itself or tied with a string. The tube is filled with water a pressed to detect any leaks. The solution to be dialysed is poured into the emptied b and another knot tied.

Suspend the bag in the beaker of water which has a hydrogen ion concentration equal to that of the gelatin solution. Allow to stand for three hours. Observe the color of the solutions inside and outside the sac.

(*a*) Are the diffusible ions equally distributed on the two sides of the membrane at the end of the experiment? Explain.

(*b*) Was the *p*H the same in the two solutions at the beginning of the experiment? Explain.

(*c*) Were equal quantities of acid and alkali needed to obtain the same color in the gelatin solution as in the water? Explain.

(*d*) Was there any diffusion of the gelatin? Test.

Experiment 22. Buffers. Prepare the following three solutions:

(*a*) **10 ml. water.**
(*b*) 10 ml. phosphate buffer, *p*H 7.0.
(*c*) 10 ml. albumin solution (egg white diluted 5 times with water).

To each add 2 drops phenolphthalein solution. Add drop by drop N/10 NaOH to each until a permanent pink color is obtained. Compare the amounts of alkali needed in each case.

Add 4 drops of methyl red to each tube and titrate with N/10 HCl until a definite red color is produced. Explain the results.

PREPARATIONS

Experiment 23. Preparation of Casein. See p. 52.

Experiment 24. Preparation of Cystine.* The basis for the isolation of cystine is its great insolubility at its isoelectric point.

Soak 100 gm. of hair† in 100 ml. of 5 per cent soap solution‡ for fifteen minutes. Stir from time to time. Decant the liquid.

Wash twice with 100 ml. of water. Discard the rinsings.

Place 200 ml. of 1:1 hydrochloric acid in a 500 ml. Florence flask and then add about 10 gm. of the hair and begin digesting on the steam bath. From time to time, as the digestion proceeds, add more of the hair until the total amount has been added. The time required should be about four hours.

When most of the hair is in solution, reflux on a sand bath until a 5 ml. sample, decolorized with norite, gives a negative biuret test (compare with a control). The refluxing should take about nine hours.

* Folin, *J. Biol. Chem.*, *8*, 9 (1910); Toennis and Bennett, *J. Biol. Chem.*, *112*, 39 (1935); Gortner and Hoffman, Organic Syntheses, *1*, 188 (1932).

† Crude sheep's wool also may be employed, but the yield is not as high (about 2 per cent). Hair obtained from barber shops generally contains matches, paper, tobacco, and similar extraneous substances which should be sorted out by hand as completely as possible. The other principal contamination is sand, which causes little trouble and need not be removed.

‡ A high grade of soap should be employed. Hair kept in hot dilute sodium carbonate solution for one to two hours yields little or no cystine. The oily material may also be removed by extracting with gasoline or benzene, but this procedure involves considerably more labor.

Filter the hot mixture and wash the insoluble residue twice with small portions of water. To the combined filtrate add 14 ml. of 40 per cent sodium hydroxide solution. Stir during the addition. Cool the mixture and add a saturated solution of sodium acetate containing 168 gm. of the salt. (A Congo red test for mineral acid should be entirely negative.) Let it stand for three days at room temperature.

Filter the precipitated cystine with suction. Dissolve the crude material in 150 ml. of *M* hydrochloric acid. Filter, and decolorize the filtrate by two to five treatments with 1 to 1.5 gm. portions of acid-washed norite. Finally filter by gravity to remove traces of solid impurities.

To the clear, almost colorless solution, add a filtered, saturated solution of sodium acetate (see above), until the Congo red reaction is negative. After it has stood five to six hours filter the cystine which crystallizes out, and wash twice with 25 to 50 ml. portions of hot water.

Allow the crystals to dry in air. Weigh. (The yield should be about 5 gm. of colorless, hexagonal plates of cystine.)

TESTS. 1. Place a few milligrams of cystine on a microscope slide, cover with a drop of concentrated ammonium hydroxide, allow the ammonium hydroxide to evaporate, and examine the residue under the microscope. Compare with the original preparation.

2. Dissolve a few milligrams of cystine in 1 ml. of saturated sodium hydroxide. Add 2 drops of 0.2 *M* lead acetate and boil for one minute. Result?

3. Place a few milligrams of cystine in a small test tube, and add 2 ml. of water and about 0.25 gm. of sodium cyanide. (**Beware!**) Shake and add 2 drops of 0.1 *M* sodium nitroprusside. Result?

Experiment 25. Preparation of Tyrosine.* Tyrosine may be prepared by acid hydrolysis or by tryptic degradation of a protein. Pancreatin, which is a commercial preparation, contains trypsin together with other peptidases needed to split native protein to amino acids. Since tyrosine is one of the few insoluble amino acids (others being cystine and leucine), it may be conveniently isolated from solution by adjusting to the proper *p*H.

Place 60 gm. of commercial casein in a liter glass-stoppered bottle and cover with 320 ml. of tap water warmed to 38°C. Shake the bottle until the casein is moistened.

Add a solution of 6 gm. of anhydrous sodium carbonate and 0.5 gm. of sodium fluoride† in 100 ml. of water warmed to 38°C.

Add a thin paste of 2 gm. of commercial pancreatin in 10 ml. of water at 38°C. Cover the mixture with a layer of 10 ml. of toluene, dilute to 600 ml., stopper, shake thoroughly, and incubate at 38°C.

After four or five days, with daily shakings, most of the casein is in solution, and chalky masses of tyrosine begin to separate. Add a second portion of 2 gm. of pancreatin in 10 ml. of water.

After twelve days cool the bottle in the icebox overnight and filter the suspension on a Buchner funnel, using several changes of filter paper.

* Organic Synthesis, *10*, 100 (1930).

† The sodium fluoride inhibits the action of oxidases.

Suspend the precipitate in 32 ml. of water, add 8 ml. of concentrated hydrochloric acid, and boil the mixture gently for thirty minutes.

Decolorize with 1 gm. of norite and filter hot. Allow the filtrate to cool.

Add a slight excess of concentrated ammonia (**Caution!**) and allow to stand overnight in the icebox.

Filter the crystalline product and wash with two 10 ml. portions of ice water.

To recrystallize, suspend in 40 ml. of water, and dissolve by adding 0.8 gm. of sodium hydroxide in 2 to 3 ml. of water. Add 0.2 gm. of norite, and filter. The residue is washed on the funnel with 2 or 3 ml. of hot water. Heat the filtrate to boiling and treat with 1.3 ml. of concentrated hydrochloric acid. Acidify to litmus with acetic acid and allow to stand overnight in the icebox.

Filter the crystalline tyrosine and wash with 15 ml. of ice-cold water.

Examine the crystals under the microscope.

Perform the Millon test on a few crystals.

(*a*) Write the formula of tyrosine.

(*b*) Why do many proteins give a positive Millon test?

(*c*) Compare the enzymatic hydrolysis with acid hydrolysis of proteins for the preparation of pure amino acids.

Experiment 26. Preparation of Glutamic Acid from Gluten. Knead thoroughly 100 gm. of flour with enough water to obtain a dough. Allow to stand for thirty minutes and add more water and repeat the extraction process. Pour off the aqueous extract which contains the starch and water-soluble proteins and repeat this process with fresh water until the aqueous extract is practically free of starch. (What test may you use?) The insoluble material is crude gluten, a mixture of several proteins.

Place about 50 gm. of gluten in a 500 ml. Florence flask and warm on the steam bath with 150 ml. of concentrated hydrochloric acid until the protein dissolves. Connect the flask with a reflux condenser and set the apparatus on a tripod. Connect the top of the condenser with a tube leading downward to an inverted funnel which just dips into a beaker of water. (Why is this necessary?) Reflux over a free flame for about eight hours. Add 5 gm. of decolorizing carbon and 200 ml. of water, heat to boiling, and filter. Evaporate the filtrate to about 50 ml. in vacuo. Transfer the solution to a 125 ml. Erlenmeyer flask and saturate with hydrochloric acid gas* while the solution is kept chilled in an ice-salt water bath. Stopper and place in a refrigerator for two or three days. Add an equal volume of cold acetone and filter the glutamic acid hydrochloride through a sintered glass funnel. Wash the precipitate with cold acetone and ether. Suck dry and hand in to your instructor.

* A convenient hydrochloric acid generator may be made by dropping concentrated sulfuric acid from a dropping funnel into a suction flask containing a mixture of concentrated hydrochloric acid and solid sodium chloride. The dropping funnel should reach below the surface of the hydrochloric acid. The side arm of the suction flask is connected to a trap containing concentrated sulfuric acid, which serves to wash and dehydrate the hydrogen chloride generated. This generator should be set up under a hood.

Experiment 27. Preparation of Ribose Nucleic Acid from Yeas Nucleoproteins are a group of cellular proteins of great importance. The tw outstanding examples are those from the thymus gland and from yeast. Th nucleoproteins may be degraded partially by splitting away the protei moiety to yield the nucleic acids. Thymonucleic acid is an example of th desoxyribonucleic acid found in the nucleus, and yeast nucleic acid is a example of the ribonucleic acid found in the cytoplasm.

Grind 50 gm. of yeast in a mortar with 125 ml. of 0.04 *M* sodium hydrox ide. Transfer to a flask with 5 ml. of the sodium hydroxide solution and hea on the steam bath for thirty minutes. Filter through a fluted filter, cool th filtrate, and just acidify with 3 *M* of acetic acid to litmus. With vigorous stir ring pour into 100 ml. of 95 per cent alcohol containing 1 ml. of concentrate hydrochloric acid. Allow the precipitate to settle, and wash twice by decan tation with 95 per cent alcohol and twice with ether. Use the ether to transfe the insoluble nucleic acid to a filter paper, and allow the precipitate to dry in the air.

Perform the biuret test on a portion of the solid. Is the test positive? Why?

Boil some of the nucleic acid with 3 *N* sulfuric acid for three minutes and perform the following tests on aliquots of the solution:

(*a*) Carbohydrates. TEST FOR RIBOSE (ORCINOL TEST). Ribose yields a characteristic green color when heated with orcinol and ferric ions in strong HCl. The method can be used to assay for ribose nucleic acid quantitatively since the ribose content of the nucleic acid is fairly constant.

Place the following solutions into the appropriate test tubes:

Tube	*Source of Ribose*	*Ferric-HCl*	*Orcinol*	*Water*
1	0.1 ml. water	3 ml.	0.3 ml.	3 ml.
2	0.1 ml. ribose (0.1 mg./ml.)	3 ml.	0.3 ml.	3 ml.
3	0.2 ml. ribose (0.1 mg./ml.)	3 ml.	0.3 ml.	3 ml.
4	5-10 mg. of ribose nucleic acid	3 ml.	0.3 ml.	3 ml.

Mix well and place into a boiling water bath for 45 minutes. Note the color.

REAGENTS. Orcinol, 10 per cent in 95 per cent ethanol.

Ferric-HCl reagent, 0.990 gm. ferric ammonium sulfate ($12\ H_2O$) dissolved in 1 liter of concentrated HCl.

Ribose standard, 10 mg. ribose dissolved in 100 ml. water.

(*b*) Purines. Add an excess of ammonium hydroxide and a few drops of 0.1 *M* of silver nitrate solution.

(*c*) Phosphates. Neutralize with ammonium hydroxide, acidify with a few drops of concentrated nitric acid, and add some ammonium molybdate solution.

QUANTITATIVE DETERMINATIONS

Experiment 28. Determination of Tyrosine in Casein.* Hydrolyze .0 gm. of casein with 20.0 ml. 6 *N* NaOH under a reflux condenser in a oiling water bath for 4 hours. Cool and add 30.0 ml. of 7 *N* H_2SO_4. Shake. Place 1.0 ml. aliquot of the hydrolysate into a clean, dry test tube. Into nother tube place 1.0 ml. of a standard tyrosine solution containing 1.0 ıg. per ml.

To each tube add 3.0 ml. of the 15 per cent $HgSO_4$ in 5 *N* H_2SO_4 and heat n a boiling water bath for 10 minutes. Cool and add to each 2.0 ml. 7 *N* H_2SO_4 and 2.0 ml. 0.2 per cent $NaNO_2$. Shake to mix and add 12.0 ml. of ater to each. Invert the tubes and filter the unknown through a dry filter aper into a dry test tube. Compare in a visual colorimeter.

Calculation:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 1.0 \times \frac{50}{1} \times \frac{100}{1000} = \text{per cent tyrosine in casein.}$$

Experiment 29. Determination of the Protein Content of Egg White. Dissolve 1.0 gm. of egg white in 50 ml. water. Shake gently and filter. Pipette 1.0 ml. of the filtrate into a test tube. Into another test tube pipette 1.0 ml. of a standard 0.25 per cent egg albumin solution. To each add 10 ml. 0.5 *M* NaOH followed by 1.0 ml. of diluted Folin reagent (1 part reagent and 2 parts water). Mix and compare in the colorimeter.

CALCULATION: (Visual colorimeter, see p. 67)

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 2.5 \times 50 \times 100 = \text{mg. protein per 100 gm. egg white}$$

Experiment 30. Determination of Nitrogen by the Kjeldahl Method. The nitrogenous substances are oxidized by boiling with concentrated sulfuric acid in a Kjeldahl flask. The nitrogen is converted to ammonia and is fixed as ammonium sulfate. Copper sulfate, mercury and selenium are some of the catalysts used in the reaction. Furthermore, potassium sulfate is frequently added to raise the boiling point of the sulfuric acid. All reagents used must be nitrogen-free and should be checked by frequent blank determinations.

The ammonia formed is liberated by making the solution alkaline with 40 per cent sodium hydroxide solution. It is then distilled into standard hydrochloric acid. The ammonia is determined by titrating the excess hydrochloric acid with standard sodium hydroxide. For the titration an indicator with a low *p*H range must be chosen to avoid interference by the buffering of the ammonium chloride in solution. Methyl red (*p*H 5.6) is a widely used indicator. One may also use alizarin or the methyl red-methylene blue indicators (see Appendix).

The usefulness of the Kjeldahl method is limited, because the nitrogen in a great many organic compounds cannot be quantitatively converted into

* Bernhart, F. W., Proc. Amer. Soc. Biol. Chem.; J. Biol. Chem., *123*, X, 1938.

ammonia by this method. Pyridine, quinoline, guanidines, nitro-, nitros azo-, diazo- and hydrazo- compounds are a partial list of the types of su stances which cannot be "Kjeldahlized" directly. However, in many cas theoretical results may be obtained if the compounds are first reduced wi zinc. Since most of the nitrogenous substances found in biological materi can be "Kjeldahlized," and because it is convenient, the method is wide used in all biochemical laboratories.

PROCEDURE. Weigh or pipette into a 500 ml. Kjeldahl flask a samp which will contain approximately 15 to 30 mg. of total nitrogen, add 0.5 gr of copper sulfate, 20 ml. of concentrated sulfuric acid, and 3 to 5 clean glas beads. Place the flask in a digestion rack or, if this is not available, clamp onto a ring stand, making a 60 degree angle with the horizontal, and put th stand in a hood. Boil the contents for one hour or longer, until all the dar color has disappeared and a clear solution is obtained. When cool, the con tents are diluted with 200 ml. of ammonia-free water. Steam out the distillin apparatus, and then place the delivery tube from the condenser into a 250 m Erlenmeyer flask containing 40.0 ml. of 0.1 *M* hydrochloric acid and 2 drop of methyl red indicator. Tilt the flask so that the delivery tube is immerse in the acid as deeply as possible.

Calculate the volume of 40 per cent sodium hydroxide which is necessar to neutralize 20 ml. of concentrated sulfuric acid.

Holding the Kjeldahl flask at a 60 degree angle, carefully pour 20 pe cent more than the calculated amount of sodium hydroxide down the neck o the flask, keeping the liquids in the flask in two layers. Attach the flask care fully to the distilling apparatus, mix the contents and distil until abou 100 ml. of distillate is collected. Lower the Erlenmeyer flask so that th delivery tube no longer dips into the standard acid. Collect another 10 ml. o distillate this way. Rinse down the outside of the delivery tube with a strea of water. Titrate the excess hydrochloric acid with standard 0.1 *M* sodiu hydroxide.

CALCULATION. $(A - B) \times 1.4 =$ milligrams of nitrogen in the sample

$A =$ Milliliters of 0.1 *M* hydrochloric acid measured into the Erlenmeye flask.

$B =$ Milliliters of 0.1000 *M* sodium hydroxide used for the back titration

REAGENTS:

Concentrated sulfuric acid, nitrogen-free
40 per cent sodium hydroxide
0.1000 M hydrochloric acid
0.1000 M sodium hydroxide
Copper sulfate, nitrogen-free
Methyl red, 0.5 per cent (in alcohol)

(*a*) Suggest another indicator.

(*b*) Could phenolphthalein be used as the indicator?

(*c*) How is the nitrogen determined in compounds which cannot b "Kjeldahlized"?

(*d*) How could one proceed to determine the ammonia in the distillat without titration?

Experiment 31. Formol Titration. During the hydrolysis of a protein the number of free carboxyl and amino groups increases. A quantitative estimation of either of these groups would be a convenient index of the degree of hydrolysis. According to the Zwitterion theory, when an amino acid in solution is titrated with alkali, it is the hydrogen ion of the ammonium group which is being titrated. Since the ammonium group of amino acids buffers in high *p*H ranges—above *p*H 11 in many cases—it is impossible to titrate them to an indicator end point. Similarly, since the carboxyl groups buffer at a low *p*H range, it is impossible to titrate them with acid to an indicator end point in water solution.

Formaldehyde added to an amino acid solution reacts with the uncharged amino group, allowing the ammonium group to buffer in a lower *p*H range and which therefore can be titrated quantitatively to the indicator end point. This is the basis for the determination of the free amino groups in protein hydrolysates. (See p. 34 for glass electrode titration of amino acids.)

Prepare 100 ml. of a 5 per cent gelatin solution by dissolving the gelatin in hot water. Cool to about 38°C., add 1 ml. of phenolphthalein and add 0.2 *M* sodium hydroxide dropwise until a definite pink color is obtained. Now add 0.1 *M* hydrochloric acid dropwise until the pink color of the phenolphthalein *just* disappears (*p*H 8.0; use a *p*H meter, if possible). Do not make this solution too acid. Transfer some of it to a narrow test tube in order to see the color change better. Set the neutralized gelatin solution in the incubator at 38°C. Prepare an active trypsin solution by dissolving 0.5 gm. of pancreatin or any other commercial trypsin powder in 25 ml. of water, add a few drops of phenolphthalein and adjust the solution with sodium hydroxide until just pink. Discharge the pink color with a drop or two of 0.1 *M* hydrochloric acid. (If a *p*H meter is available, adjust the solution to *p*H 8.0.) At "zero" time add the trypsin solution to the gelatin solution and mix.

Immediately after the addition of the enzyme solution, pipette two 10 ml. portions of the digest into 100 ml. beakers and boil to destroy the enzyme. Note the time. Continue boiling gently for two minutes. Cool. To each sample add 15 ml. of neutralized formalin solution* and 3 drops of phenolphthalein.

Titrate each sample with 0.02 *M* sodium hydroxide to a pink end point. At intervals of fifteen, thirty, sixty, ninety and 120 minutes remove two 10 ml. samples from the digest and treat as described for the original samples, which serve as control values and are to be deducted from any succeeding values obtained.

Plot the volume of alkali (ordinate) against time in half-hour intervals (abscissa). Bearing in mind that 1 ml. of 0.1 *N* sodium hydroxide is equivalent to 1.4 mg. of amino acid nitrogen, plot the milligrams of amino acid nitrogen (ordinate) against time (abscissa).

* Formalin solution (to be prepared when needed and kept in a glass-stoppered bottle): Filter the stock formalin solution (37 to 40 per cent formaldehyde) through a fluted filter. Dilute 40 ml. of this solution with 80 ml. of water and neutralize by adding 0.02 *M* sodium hydroxide from a burette until the color is just pink to phenolphthalein. (**Caution!** Use a 10 ml. graduated cylinder to measure out the formula. A pipette may be used if it is attached to a guard tube containing activated carbon.)

(*a*) Why must alkali be added to the formalin solution until just p to phenolphthalein?

(*b*) Why is it necessary to use recently distilled formalin?

(*c*) Why must formalin be kept stoppered?

(*d*) Why is sodium carbonate added to the casein mixture?

(*e*) What is the *p*H of the mixture when the acid is added to the case sodium carbonate until the pink color of phenolphthalein is just discharge

(*f*) What is the purpose of attaining this *p*H?

Experiment 32. Amino Nitrogen Determinations (Van Slyke).* T compound containing the amino group is allowed to react with a soluti

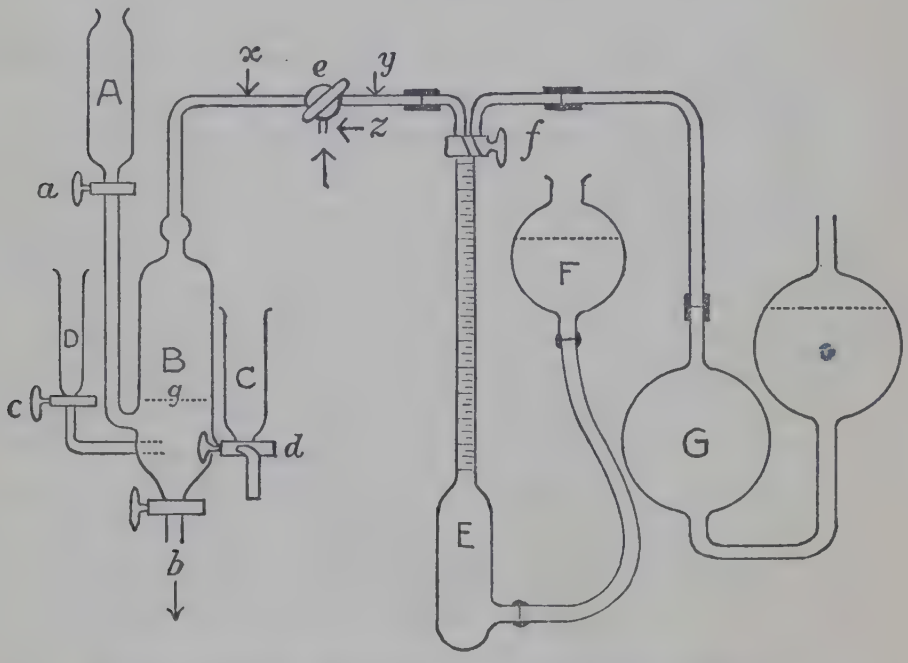


Fig. 6.—The Van Slyke micro amino nitrogen apparatus.

of nitrous acid in a special apparatus, and the volume of the resulting nitroge gas is measured.

PROCEDURE. The apparatus should always be left filled with water an all stopcocks closed. Drain the water from the apparatus, with the exceptio of the leveling bulb and chamber *E* (Fig. 6) up to but not beyond stopcock Close all stopcocks, but have *x* to *z* open.

To *A* add 5 ml. of glacial acetic acid, open *a* and allow the acid to flo into *B*. Close *a* and add 12 ml. of 30 per cent sodium nitrite solution to *A* Open *a* and allow the nitrite solution to flow into *B* and mix with the aci until the mixture just flows out at *z;* then quickly turn *e,* so that *x* to *y* i connected, allowing as little liquid to escape as possible.

With *a* open, shake the reaction chamber vigorously until the level of th

* Van Slyke, *J. Biol. Chem., 16,* 121 (1913) ; *23,* 407 (1915).

The ninhydrin reaction has been adapted as a quantitative determination of amin acids in the Van Slyke manometric gas apparatus. MacFayden, *J. Biol. Chem., 145,* 38 (1942).

solution falls to *g*. Stop the shaking and open *x* to *z*, so that the accumulated gases in *B* are removed and the solution just passes through *e*. Then quickly turn *e* so that *x* to *y* is connected. Open stopcock *c* momentarily to remove any trapped gas. Repeat this shaking down and gas removal procedure twice again.

After the solution has been shaken down to level *g* for the third time,

Table 2

MILLIGRAMS OF AMINO N CORRESPONDING TO 1 ML. OF N_2 GAS AT 11°–30°C.; 728–772 MM. PRESSURE

t°	728	730	732	734	736	738	740	742	744	746	748	750
11	.5680	.5695	.5710	.5725	.5745	.5760	.5775	.5790	.5805	.5820	.5840	.5855
12	.5655	.5670	.5685	.5700	.5720	.5735	.5750	.5765	.5780	.5795	.5815	.5830
13	.5630	.5645	.5660	.5675	.5695	.5710	.5725	.5740	.5755	.5770	.5785	.5805
14	.5605	.5620	.5635	.5650	.5665	.5680	.5700	.5715	.5730	.5745	.5760	.5775
15	.5580	.5595	.5610	.5625	.5640	.5655	.5670	.5685	.5705	.5720	.5735	.5750
16	.5555	.5570	.5585	.5600	.5615	.5630	.5645	.5660	.5675	.5690	.5710	.5725
17	.5525	.5540	.5555	.5575	.5590	.5605	.5620	.5635	.5650	.5665	.5680	.5695
18	.5500	.5515	.5530	.5545	.5560	.5580	.5595	.5610	.5625	.5640	.5655	.5670
19	.5475	.5490	.5505	.5520	.5535	.5550	.5565	.5580	.5595	.5610	.5630	.5645
20	.5445	.5460	.5475	.5495	.5510	.5525	.5540	.5555	.5570	.5585	.5600	.5615
21	.5420	.5435	.5450	.5465	.5480	.5494	.5510	.5525	.5540	.5555	.5575	.5590
22	.5395	.5410	.5425	.5440	.5455	.5470	.5485	.5500	.5515	.5530	.5545	.5560
23	.5365	.5380	.5395	.5410	.5425	.5440	.5455	.5470	.5485	.5500	.5515	.5530
24	.5335	.5350	.5365	.5380	.5400	.5415	.5430	.5445	.5460	.5475	.5490	.5505
25	.5310	.5325	.5340	.5355	.5370	.5385	.5400	.5415	.5430	.5445	.5460	.5475
26	.5260	.5295	.5310	.5325	.5340	.5355	.5370	.5365	.5400	.5415	.5430	.5445
27	.5250	.5265	.5280	.5295	.5310	.5325	.5340	.5355	.5370	.5385	.5400	.5415
28	.5220	.5235	.5250	.5265	.5280	.5295	.5310	.5325	.5340	.5355	.5370	.5385
29	.5195	.5210	.5220	.5235	.5250	.5265	.5280	.5295	.5310	.5325	.5340	.5355
30	.5160	.5175	.5190	.5205	.5220	.5235	.5250	.5265	.5280	.5295	.5310	.5325

t°	752	754	756	758	760	762	764	766	768	770	772
11	.5870	.5885	.5900	.5915	.5935	.5950	.5965	.5980	.5995	.6010	.6030
12	.5845	.5860	.5875	.5890	.5905	.5925	.5940	.5955	.5970	.5985	.6000
13	.5820	.5835	.5850	.5865	.5880	.5895	.5910	.5930	.5945	.5960	.5975
14	.5790	.5805	.5825	.5840	.5855	.5870	.5885	.5900	.5915	.5935	.5950
15	.5765	.5765	.5795	.5810	.5830	.5845	.5860	.5900	.5875	.5890	.5905	.5920
16	.5740	.5755	.5770	.5785	.5800	.5815	.5830	.5850	.5865	.5880	.5895
17	.5710	.5730	.5745	.5760	.5775	.5790	.5805	.5820	.5825	.5850	.5865
18	.5685	.5700	.5715	.5730	.5745	.5765	.5780	.5795	.5810	.5825	.5840
19	.5660	.5675	.5690	.5705	.5720	.5735	.5750	.5765	.5780	.5795	.5810
20	.5630	.5645	.5660	.5675	.5690	.5705	.5725	.5740	.5755	.5770	.5785
21	.5605	.5620	.5635	.5650	.5665	.5680	.5695	.5710	.5725	.5740	.5755
22	.5575	.5590	.5605	.5620	.5635	.5650	.5665	.5680	.5695	.5715	.5730
23	.5545	.5560	.5575	.5595	.5610	.5625	.5640	.5655	.5670	.5685	.5700
24	.5520	.5535	.5550	.5565	.5580	.5595	.5610	.5625	.5640	.5655	.5670
25	.5490	.5505	.5520	.5535	.5550	.5565	.5580	.5595	.5610	.5625	.5640
26	.5460	.5475	.5490	.5505	.5520	.5535	.5550	.5565	.5580	.5595	.5610
27	.5430	.5445	.5460	.5475	.5490	.5505	.5520	.5535	.5550	.5565	.5580
28	.5400	.5415	.5430	.5445	.5460	.5475	.5490	.5505	.5520	.5535	.5550
29	.5370	.5385	.5400	.5415	.5430	.5445	.5460	.5475	.5490	.5505	.5520
30	.5340	.5355	.5370	.5385	.5400	.5415	.5430	.5445	.5460	.5475	.5490

close *a*, lower *F* and open *f* so that *B* communicates with *E*. Shake the reaction chamber for three minutes.

Open *a* so that the solution flows up to and just into *E*. Close *f*. Raise the bulb *F* and open *f*, so that *E* to *G* is connected and the water forces all the gases through the capillary tubes and well into the Hempel pipette *G*. (Admission of a little water into *G* does no harm.) Close *f*. Connect the shaking arm to *G* and slowly shake the pipette for one minute. Lower *F* and carefully open *f* so as to force the gas from *G* back into *E*, until the lower level of the permanganate solution just comes to the zero mark on the upper part of *E*. Close *f*. Raise *F* so that the level of the water in the bulb is the same

as that in *E*. Read the volume of the blank. This volume should never exce 0.1 ml. and is usually 0.07 ml.

Turn *e*, so that *z* to *y* is connected and rinse out the gas and permangana solution. (Run some permanganate from *G* into *E* during the process Open *a* and, keeping *e* open so that *x* to *y* is connected, vigorously shake t reaction vessel until the nitrite solutions falls to level *g*.

Close *a*, lower the bulb *F* and open *f* so that *y* to *E* is connected. Place t unknown solution in *C* and open *d*, so that the meniscus of the solutio reaches the 2 ml. mark on *C*, allowing the excess to flow into a beaker. Tu *d* so that *C* connects with *B*, allowing the solution to flow into *B* and dow to the zero mark in *C*. Close *d*.

Vigorously shake the reaction vessel for three minutes. Open *a* so tha the solution flows up to and just into *E*. Close *f*. Raise the bulb *F* and open so that *E* to *G* is connected and the water forces all the gases through th capillary tubes and well into *G*. Close *f*. Slowly shake the Hempel pipette fo one minute. Lower *F* and, carefully opening *f*, bring the gas from *G* into until the lower level of the permanganate solution just comes to the zer mark on the upper part of *E*. Close *f*. Raise *F* so that the level of the water i the bulb is the same as that in *E*. Read the volume of the gas.

(When foaming solutions are used, add a drop of caprylic alcohol int *B* through *D*.)

CALCULATIONS. The milliliters of nitrogen obtained in the blank deter mination are subtracted from the milliliters of nitrogen obtained in th determination, and this value is multiplied by the temperature and pressur factor, obtained from Table 2. The resulting value represents the milligram of amino nitrogen in 2 ml. of the unknown solution.

REAGENTS:

ALKALINE PERMANGANATE REAGENT. Dissolve 50 gm. of potassium per manganate and 25 gm. of potassium hydroxide in 1 liter of water.

SODIUM NITRITE REAGENT. Dissolve 30 gm. of sodium nitrite in 100 ml of water and filter the resulting solution if necessary.

Experiment 33. Titration of an Amino Acid, Using the Glass Electrode

Among the various ways of determining the *p*H of a solution are the well known colorimetric method (see p. 51, Experiment 10, for directions when using colored glass standards), the hydrogen electrode method and the glass electrode method.

The hydrogen electrode is the standard method to which all others are referred, but it is fraught with difficulties and is not always satisfactory. Aside from the elaborate electrical system, many substances interfere with its use. Among these are sulfur compounds, proteins and oxidizing agents.

The glass electrode was developed to circumvent these difficulties. It is a thin membrane of soft glass of special composition, usually taking the form of a bulb blown at one end of a tube of the glass. The bulb is filled with 0.1 *N* hydrochloric acid. Sealed into the other end of the tube is a silver-silver chloride electrode which dips into the enclosed acid.

Within the limits of its use it would appear that the glass membrane is

permeable only to hydrogen ions. When interposed between two solutions of different *p*H, a potential develops. One solution is the 0.1 *N* hydrochloric acid within the electrode, the other is the unknown. Then

$$E = \frac{RT}{F} (pH_1 - pH_2)$$

Accordingly, if the glass electrode is immersed in 0.1 *N* hydrochloric acid, no potential should develop. This is not always true. The potential, called the *asymmetry potential,* which may develop is thought to be due to strains in the glass, since it is large when the electrodes are first blown, becoming smaller in time.

The advantages of the glass electrode are its small size, requiring but a small sample of the material to be tested; its lack of contamination of that sample; its ability to function in any solution which does not attack glass—including nonaqueous solutions. It functions reproducibly in the acid range and is satisfactory up to a *p*H of 9 or 10, beyond which special glasses are available to extend its range still higher.

Although the glass membrane is thin, careful handling or protection of it by a bell will prevent breakage. The membrane should not be allowed to dry out. When not in use it should be kept immersed in water.

A saturated calomel cell is usually used in conjunction with the glass electrode. Because of the high electrical resistance of the glass membrane, the usual potentiometer-galvanometer circuit cannot be used to measure the potential which is developed. However, a number of satisfactory circuits have been designed in which vacuum tube amplifiers are inserted in a potentiometer circuit. These may be powered from dry-cell batteries or, in some cases, directly from the line. A direct reading meter is used instead of a galvanometer; if its response is linear, it may be calibrated in *p*H units. Since the temperature factor is important in obtaining correct results, many instruments incorporate a variable resistance in the circuit so that temperature corrections are automatically made on setting a dial.

In general, the procedure for use is as follows: The instrument is turned on ten to fifteen minutes before use to allow it to reach equilibrium. It is then calibrated. This must be done each time the instrument is operated, since the settings are not constant because of the asymmetry potential and other electrical and mechanical variables. The glass and the calomel electrodes are immersed in a standardized buffer solution (usually at a *p*H around 4). The temperature dial is adjusted. Other dials are then turned until the meter reads the *p*H of the buffer. The electrodes are rinsed, and the process is repeated with another buffer in the alkaline range. It is sometimes advisable, for accurate work, to repeat with a buffer intermediate between the two first used. Adjustment is finally achieved so that the meter will indicate *p*H directly. (A check on this should be made occasionally, since the meter readings are inclined to drift.) In some models the calibration is performed by means of a standard cadmium cell incorporated within the unit. Finally, the electrodes are rinsed again, immersed in the solution whose *p*H is to be determined, and a direct reading is taken from the meter.

For further details, the student is advised to consult the literature suppli with each commercially available instrument.

The titrations are usually carried out in dilute solution—0.05 *M* of t amino acid, with strong acid or base (1 *M*)—so that the volume chan during the course of the titration (5 per cent) has but a negligible effect the dissociation of the amino acid. It is essential to obtain a water blank the same time. This is the volume of acid or base needed to bring the *p*H the water itself to the levels reached during the titration of the amino aci At high and low *p*H the water blank is considerable, and correction must made for it.

From the titration curves, corrected for water blank, the apparent dissoci tion constants, K^1, of the amino acid can be calculated, using the Henderso Hasselbach equation. For the calculation of the "true" dissociation constan K, the student is referred to Chapter 25 in Clark's *Determination of Hydroge ions* (third edition, Williams and Wilkins).

Into each of two 200 ml. beakers, weigh 0.375 gm. of glycine (0.005 *M*) Dissolve each charge in 100 ml. of freshly boiled, cool distilled water. Sti the solutions until homogeneous and measure the *p*H of one of them with *p*H meter. Using a calibrated 10 ml. microburette graduated in 0.01 ml., ad standard 1 *N* hydrochloric acid in 0.25 ml. portions until 7 ml. have bee added, stirring well and measuring the *p*H after each addition.

Repeat the titration in an identical manner on the second glycine charge using 1 *N* sodium hydroxide for the titration.

To determine the water blank, measure the *p*H of 100 ml. of the sam water used for the previous titrations. Add 1 *N* hydrochloric acid in incre ments of 0.01 ml. until 0.1 ml. has been added; then in increments of 0.05 ml until 0.5 ml. has been added. Repeat the titration on a fresh sample of 100 ml. of water, using 1 *N* sodium hydroxide.

Plot on the same graph the titration curve of glycine and the water blank titration.

Draw a corrected titration curve of glycine by subtracting the water blank correction for ten points on both sides of the curve.

Calculate pK_1^1 (the carboxyl group) and pK_2^1 (the amino group), using five points for each constant.

(*a*) How do the constants you determined compare with those reported in the literature?

(*b*) What may be the reason for any discrepancy?

Experiment 34. Electrometric Formol Titration. Adjust 500 ml. of 15 per cent formaldehyde solution to the same *p*H you obtained with the glycine dissolved in water in the previous experiment. Weigh two charges of glycine as before, but instead of water dissolve each charge in 100 ml. of the neutralized 15 per cent formaldehyde solution. Measure the *p*H of one of the solutions with the *p*H meter. Titrate with 1 *N* hydrochloric acid as in the previous experiment. Repeat the titration with 1 *N* sodium hydroxide on the second charge. Determine the "water blank" with acid and alkali on two 100 ml. portions of the neutralized formaldehyde solution.

Plot these titration curves on the same graph as the titration curves in water. Draw a corrected formol titration curve.

(*a*) Which branch of the curve is more affected by the formaldehyde?
(*b*) What is the dissociating group affected by the formaldehyde?
(*c*) Does formaldehyde remove completely the buffering capacity of the dissociating group under (*b*)?

Chapter 4 **Enzymes***

Enzyme activity is influenced by many factors, such as *p*H, temperature, co centration of the substrate, concentration of the enzyme, concentration of t products of the reaction, inorganic salt concentration, activators and i hibitors. Each of these factors must be carefully controlled to obtain resu which can be duplicated.

In expressing enzyme activity, a convenient arbitrary unit is selected, a preparations are described in terms of the unit.

Experiment 1. Preparation of Potato Extract. Wash, peel and gra a potato. Transfer the gratings and adhering liquid to a piece of cheeseclot Fold the cloth into a bag and, holding the top ends of it, move it up an down through 100 ml. of water contained in a beaker. Continue this for thre to four minutes. Test the pulp for protein (p. 18). Notice the substanc which settles to the bottom of the beaker.

Filter the supernatant liquid and use a portion of the filtrate for each o the following tests: (*a*) reducing sugar (p. 2) and (*b*) proteins (p. 18).

(*a*) What ions are present in the extract?
(*b*) What substance settles out?

Experiment 2. Oxidase. To each of two test tubes add 5 ml. of th filtrate from Experiment 1. Place one of these tubes in a boiling water bat for ten minutes, remove it, and cool. Add to each of the two tubes 0.5 ml. o phosphate buffer, *p*H 6, and 2 ml. of freshly prepared 0.2 per cent catecho solution. Shake each tube thoroughly for several minutes. Let stand fo thirty minutes.

(*a*) How do the boiled and unboiled extracts differ in their action o catechol solution? Why?

(*b*) Describe the color changes that take place in the enzymatic oxidatio of catechol.

(*c*) What is a possible oxidation product of catechol?

Experiment 3. Sucrase. Place a cake of yeast (or its equivalent) in mortar and add about 5 gm. of sand. (It is preferable not to use the dehy drated yeast preparations, because these yield milky solutions which mas some of the reactions. If brewers' yeast is available, use about 10 gm.) Ad 10 ml. of toluene and grind the mixture. Add 30 ml. of water in 3 ml. por tions, grinding the mass thoroughly. During fifteen minutes grind the sus pension occasionally and then centrifuge it for five to ten minutes. Pipett off the supernatant fluid, which will be clear or milky, depending on whethe

* Other experiments on enzymes will be found under saliva, milk, gastric and pan creatic digestion, and bile.

ındehydrated or dehydrated yeast preparation was used. (The grinding may ɔe done in a Waring blendor.)

Set up the following mixtures in test tubes:

	Yeast Supernatant	*Acetate Buffer* pH 4.4	*Fresh 1 Per Cent Sucrose*	*1 Per Cent Starch*	*Water*
1.....	3 ml.	1 ml.	..	..	3 ml.
2.....	3 ml.	1 ml.	3 ml.		
3.....	3 ml.	1 ml.	..	3 ml.	
4.....	3 ml. boiled for 1 minute	1 ml.	3 ml.		

Mix well the contents of the tubes and allow to stand at room temperature for thirty minutes. Make the solutions just alkaline to litmus with 1 *M* sodium carbonate solution, and perform the Benedict test on each of them.

Experiment 4. The Precipitation of Sucrase by Alcohol. Add 10 ml. of the sucrase extract to 40 ml. of 95 per cent alcohol. Allow to stand until a precipitate forms. Centrifuge the mixture. Discard the supernatant. Dissolve the residue in the centrifuge tube in 5 ml. of water. Centrifuge down any insoluble debris if necessary. Test the supernatant for sucrase activity.

(*a*) Why is toluene used in the preparation of sucrase from yeast?

(*b*) What does this experiment show with regard to the specificity of sucrase action?

(*c*) Is sucrase soluble in alcohol?

(*d*) How would the alcoholic supernatant behave towards sucrose?

(*e*) Why is it necessary to dissolve the alcoholic precipitate in water before testing for sucrase action?

(*f*) Write the equation for the hydrolysis of sucrose.

(*g*) What is meant by "inversion"?

Experiment 5. Carbonic Anhydrase. This enzyme is widely distributed. It occurs in red blood cells, brain and other tissues. It catalyzes the equilibrium between carbon dioxide, water and carbonic acid.

$$CO_2 + H_2O \leftrightharpoons H_2CO_3$$

Add 1 ml. of 0.04 per cent bromthymol blue to 25 ml. of water in a large test tube. Place a clean thermometer into the solution and cool the tube in an ice bath. When the temperature of the solution drops to 4°C. add 1.0 ml. of 5 per cent sodium bicarbonate solution. Insert a capillary tube through which carbon dioxide is passed into the solution. (Adjust the carbon dioxide flow so that it is not too violent by first passing the gas through a control test tube containing 25 ml. of water.) Start a stop watch when the carbon dioxide starts bubbling through the bicarbonate solution. Record the time elapsed until a sharp color change from blue to yellow-green occurs. Repeat the experiment exactly, but add 0.1 ml. of blood (whole or defibrinated) before inserting the carbon dioxide-bearing capillary.

(*a*) Is there a difference in the time elapsed before the color change?

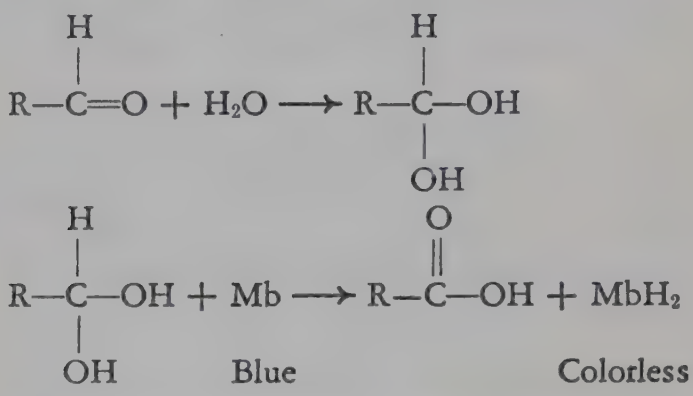
(*b*) What is the difference due to?

(*c*) What causes the color change of the indicator?
(*d*) What is the physiologic function of carbonic anhydrase?

Experiment 6. Schardinger Enzyme. Milk contains an enzym Schardinger—which catalyzes the oxidation of a variety of aldehydes acids. The reaction proceeds anaerobically and can be demonstrated i

Fig. 7.

suitable hydrogen acceptor such as methylene blue is present. The reacti is formulated via an intermediate hydrated aldehyde as follows:



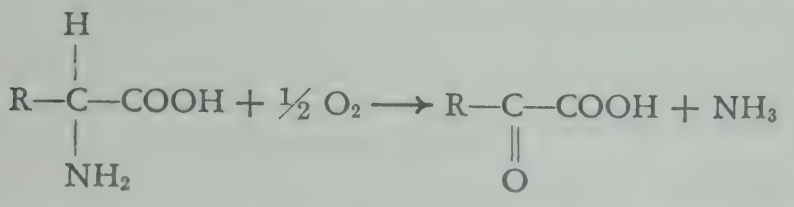
The progress of the reaction can be noted from the change of the bl oxidized form of methylene blue to its colorless reduced form.

The reaction is conveniently carried out in a vessel designed for the stu of anaerobic reactions, the Thunberg tube (Fig. 7).

Place 5 ml. of milk into each of three Thunberg tubes. Heat the conten of one for one minute in a boiling water bath. Add 1 ml. of 0.02 per ce methylene blue to each of the tubes. Pipette 1 ml. of 0.5 per cent neutr formaldehyde solution into the hollow stopper of the tube with the boil milk and into one of the other stoppers. Into the third stopper pipette 1 m of water. Grease the stoppers and insert them into the appropriate tub without splashing any of their contents into the tubes. Turn the stoppe so that the hole communicates with the side arm. Cool in ice water. Evacua the tubes on a suction pump and turn the stoppers to seal them before di connecting the suction line. Place the tubes into a 40°C. water bath. Aft five minutes, tilt the tubes, causing the contents of the stoppers to flow in the tubes. Continue the incubation at 40°C. and note the time of comple decolorization.

(*a*) Which tube became decolorized first?
(*b*) Is methylene blue the only substance in the tube that is reduced?
(*c*) Compare the oxidase systems in Experiment 2 and in this.

Experiment 7. D-Amino Acid Oxidase.* Liver and kidney contain enzymes which oxidize amino acids according to the following reaction:



There are apparently two separate enzyme systems, one of which acts on amino acids having the L-optical configuration, and the other on the amino acids of the D configuration. The latter, the D-amino acid oxidase, is more stable and is easily extracted either from fresh kidney or a dry preparation of whole kidney.†

The extract from whole kidney is made up for a class of twenty students as follows: Cut four fresh lamb kidneys lengthwise. Remove the sheath and excise most of the fat with scissors. Cut the kidneys into pieces the size of a pecan and place them into a Waring blender. Put the lid on the Waring blender and run the machine until the tissue is reduced to a thick paste. Do not allow the tissue to become warm by running the machine too long. Pour the tissue paste, usually called "homogenate," into 2 liters of acetone. Pour the paste slowly and stir the acetone vigorously.

Filter the greyish mass on a Buchner funnel. Wash the mass four times with 200 ml. of acetone. Air dry the powder on the Buchner funnel and then place it in a vacuum desiccator over P_2O_5. Change the P_2O_5 if necessary several times during a week. Since the product of the amino acid oxidation is determined with a ketone reagent, the enzyme preparation must be acetone-free. To test for the absence of acetone, grind 0.5 gm. of the grey powder with 10 ml. of water. Add to the suspension 5 ml. of 30 per cent trichloracetic acid solution and filter after ten minutes. Add to 2 ml. of the filtrate 2 ml. of a saturated 2:4-dinitrophenylhydrazine solution in 2 *N* hydrochloric acid. If a precipitate forms, all the acetone had not been removed, and therefore continue the drying of the main product over P_2O_5 in vacuo.

When the "acetone powder" is acetone-free, it can be used for enzyme studies by each student.

For the extraction of the enzyme, proceed as follows: Grind up 5 gm. of either the "acetone powder" or Viobin whole kidney powder with 5 gm. of sand and 150 ml. of water. After thorough grinding, filter through fluted filter paper. Use three funnels to speed up the collection of the filtrate. Dissolve 1 gm. of D-L alanine in 50 ml. of 0.1 *M* pyrophosphate buffer solution of *p*H 8.3. Mix the alanine solution with 50 ml. of the enzyme extract in a 500 ml. Erlenmeyer flask. Add 1 ml. of 0.1 *M* arsenious acid solution (see

* Warburg and Christian, *Biochem. Z., 298*, 150 (1938); Krebs, *Z. Physiol. Chem., 217*, 216 (1933); Krebs, *Biochem. J., 29*, 1620 (1935).

† Obtainable from the Viobin Corporation, Monticello, Illinois.

Appendix) and incubate the solution at 37°C. for twelve hours. It is pre able, but not essential, to bubble oxygen or air through the solution dur the incubation.

At the end of the incubation period, add 15 ml. of freshly prepared per cent trichloracetic acid solution and mix the contents of the flask. A ten minutes, filter free of the precipitated proteins. Add to the filtrate 20 of a saturated solution of 2:4 dinitrophenylhydrazine in 2 *N* hydrochlo acid (see Appendix) and place the mixture in an icebox for twelve hou Filter the precipitated 2:4 dinitrophenylhydrazine of pyruvic acid. T product of several students' experiment may be pooled, recrystallized, a the melting point compared with that of an authentic sample of the 2 dinitrophenylhydrazine of pyruvic acid (213°).

(*a*) How could you prove that this enzyme preparation oxidizes on D-alanine?

(*b*) Calculate the volume of oxygen at standard temperature and pressu needed for the oxidation of the D-amino acid in this experiment.

(*c*) Devise an experiment in which the ammonia liberated during t course of this reaction could also be determined.

Experiment 8. Catalase Activity.

(*a*) Into a 250 ml. Erlenmeyer flask, introduce 90 ml. of *M*/150 hydroge peroxide and 10 ml. of a mixture of equal parts of *M*/15 disodium phospha and *M*/15 monopotassium phosphate. Place in an ice-water bath at 2°.

(*b*) At "zero" time add 10 ml. of the enzyme solution (1:250 Worthingto Catalase). Mix.

(*c*) At 10 minutes add 20 ml. of 20 per cent sulfuric acid and titrate wit 0.05 *N* $KMnO_4$ to a permanent rose color.

(*d*) Repeat using 10 ml. of water instead of the enzyme solution.

(*e*) Subtract the quantity of permanganate in the enzyme experimer from that obtained in the control experiment. This gives the quantity hydrogen peroxide decomposed by the enzyme. One ml. of 0.05 *N* $KMnO_4$ equivalent to 0.4 mg. or 0.28 ml. of available oxygen. Express the catala activity in terms of mols H_2O_2 decomposed per ml. of enzyme solution p hour.

(*f*) Repeat using 10 ml. of enzyme which had been boiled for 30 secon and cooled.

(*g*) Repeat using 10 ml. of enzyme solution in the presence of 1 ml. 0.1 *M* mercuric chloride solution.

Experiment 9. Quantitative Estimation of Pepsin Activity.* In th hemoglobin method for the estimation of pepsin, hemoglobin is digeste under standard conditions at an optimum *p*H of 1.6. The undigested hem globin (large molecular components) is precipitated with trichloracetic aci and the hydrolytic products (polypeptids) are estimated in the filtrate wit the Folin phenol reagent, which gives a blue color, due mostly to the tyrosin and tryptophan groupings.

* Northrop, *Crystalline Enzymes* (1939), 153.

PREPARATION OF HEMOGLOBIN SUBSTRATE.* Centrifuge 25 ml. of defibrinated blood for twenty to thirty minutes. The serum and white cells are siphoned off, and the red cells are mixed with an equal volume of 1 per cent sodium chloride solution. The mixture is centrifuged and the supernatant siphoned off and discarded. The red cells are mixed with three times their volume of distilled water and shaken gently to hemolyze the cells. The mixture is centrifuged once again and the clear hemoglobin solution retained.

ESTIMATION OF HEMOGLOBIN CONCENTRATION. Follow the directions for the determination of the hemoglobin by the Wong iron method (page 122, Experiment 20). Use 1.0 ml. of hemoglobin solution in place of 0.5 ml. of blood. Read in the Klett photocolorimeter, using the 54 filter and setting the blank at zero. Calculate the hemoglobin concentration. Dilute the hemoglobin concentration so that it is exactly 2.5 gm. per cent. Add 1 gm. of merthiolate to each 50 ml. of solution (as a preservative). Place in a tightly stoppered flask in the refrigerator.

(*a*) Prepare two pepsin solutions by dissolving 100 mg. of each (1) Merck USP pepsin and (2) Stomach lining powder, Viobin, in 100 ml. water. If they do not dissolve completely, use as a suspension.

(*b*) Prepare 4 test tubes labeled a_1, a_2, b_1 and b_2. To each add 4 ml. of 2.5 per cent hemoglobin solution and 1 ml. of 0.3 *N* HCl.

(*c*) At "zero" time add 1 ml. pepsin (1) to tube a_1. Mix. At exactly 2 minutes add 1 ml. pepsin (2) to tube b_1. Mix.

(*d*) At exactly 10 minutes add 10 ml. 5 per cent trichloracetic acid to tube a_1. Mix. At exactly 12 minutes add 10 ml. 5 per cent trichloracetic acid to tube b_1. Mix.

(*e*) Add 10 ml. 5 per cent trichloracetic acid to tubes a_2 and b_2 followed by 1 ml. pepsin (a) to tube a_2 and 1 ml. pepsin (b) to tube b_2. These will serve as controls since the enzymes are destroyed by the trichloracetic acid.

(*f*) Filter the contents of tubes a_1, a_2, b_1 and b_2 using Whatman #2 dry filter paper into 4 clean labeled test tubes.

(*g*) Transfer 5 ml. of each of the filtrates to each of 4 labeled test tubes. Prepare a standard tube containing 5 ml. of a tyrosine solution containing 0.02 mg. tyrosine per ml.

(*h*) To each tube add 10 ml. 0.5 *N* NaOH and 3 ml. of Folin reagent previously diluted 3 times with water. Mix. Compare the unknowns against the standard in a colorimeter.

CALCULATION: (Visual colorimeter, see p. 67)

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.1 \times \frac{16}{5} = \text{mg. "tyrosine equivalents" per mg. enzyme}$$

(Note: the reading of tubes a_2 and b_2 are subtracted from a_1 and b_1 respectively if the standards are set at the same height. If not, subtract the calculated "tyrosine equivalents.")

* A powdered soluble hemoglobin, supplied by National Biochemical Corp., Cleveland 28, Ohio, can also be used.

Experiment 10. Yeast Fermentation. The fermentation reacti sometimes called glycolysis, is one of the oldest known to biochemistry. mixture of enzymes in yeast, responsible for what is in reality a series many reactions, was originally called "zymase." In this reaction glucose eventually converted to ethanol or acetic acid and carbon dioxide. In or to follow the course of such a reaction quantitatively the manometric tech of Barcroft and Warburg is used (see p. 45). The following experiment designed to allow the student to study a gas-yielding reaction with a ve much simplified version of the aforementioned apparatus and gives quali tive results.

(*a*) Construct two manometers from glass capillary tubing and insert t short end of each into a rubber stopper designed to fit a 50 ml. Erlenmey flask (see Fig. 8). Push a #20 hypodermic needle through the stopper

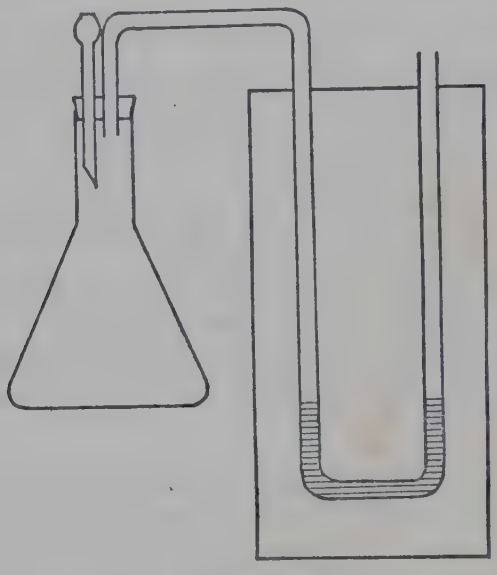


Fig. 8.

a point adjacent to the manometer tubing. Add indicator fluid to th manometer by dipping the long end into a dye solution (Evans blue) an withdraw 2 to 3 inches. Turn the manometer to an upright position so th the fluid fills the bottom U of the manometer. Fix a piece of graph pap behind the manometer and secure with scotch tape.

(*b*) Prepare a Krebs-Ringer-phosphate solution by mixing the followin solutions in order: 100 ml. NaCl (0.154 *M*), 4 ml. KCl (0.154 *M*), 3 m $CaCl_2$ (0.11 *M*), 1 ml. KH_2PO_4 (0.154 *M*), 1 ml. $MgSO_4$ (0.154 *M*) an 12 ml. of a sodium phosphate buffer, *p*H 7.4 (0.1 *M*).

(*c*) Homogenize 1 gm. of dried yeast powder with 100 ml. of the Krel solution for about 2 minutes. Dissolve 1 gm. of glucose in the suspension an pipette 10 ml. of it into each of two 50 ml. Erlenmeyer flasks. To one fla add 0.2 ml. water and to the other add 0.2 ml. of a solution of $HgCl_2$ (0.1 *M*

(*d*) After 5 to 10 minutes, stopper the open ends of the hypoderm needles (wax or apiezon cement) and mark the starting level in the ope limb of each manometer quickly.

(*e*) At intervals of 1 minute gently shake each solution and mark th level of the indicating fluids again. Record the changes.

Explain the results.

What other inhibitors may be used?

Improvise and perform another experiment using this apparatus after consulting with your instructor.

Experiment 11. Tissue Respiration Measurements. Enzyme activity has been studied until now in solutions free of intact cells. This procedure is open to the criticism that the enzyme is present under artificial and unphysiologic conditions and that many coenzymes are lost upon disruption of the cell wall. It is obviously impossible to measure most enzyme activities in the intact living animal (in vivo). A compromise procedure has been worked out due mainly to the contributions of Barcroft and Warburg. Tissues, quickly removed from the sacrificed animal, are sliced very thin (0.5 mm.) so that two conditions are met: (*a*) Intact cells are present in a great proportion of the total weight of the slice; and (*b*) when placed in a medium containing a suitable substrate and saturated with dissolved oxygen, there is rapid and adequate movement of oxygen and substrate into the deepest cells and adequate removal of carbon dioxide and waste metabolites into the solution. The respiration flask containing the tissue slices, medium, gas and alkali (for absorption of carbon dioxide) is connected to a manometer which is capable of measuring the gas exchange quantitatively (see Fig. 9). (For details of the construction of various types of vessels, manometers and shaking thermostats, and for the methods of calibration of the vessels and manometers, reference is made to *Manometric Techniques* by Umbreit et al., Burgess Publ. Co., 1945, Minneapolis, Minn., and *Manometric Methods* by M. Dixon, 1943, Macmillan, N. Y.)

Oxygen Utilization by Rat Liver Slices. The following experiment is designed to illustrate to the student one determination which is possible using this technic. Other determinations will be found in the references cited. Prepare the following respiration vessels:

Flask	*1*	*2*	*3*	*4*	*5*
(*a*) Main compartment:					
Ringer-phosphate-glucose*	2.4 ml.	2.0 ml.	2.0 ml.	2.0 ml.	2.0 ml.
(*b*) Side-arm:					
Ringer-phosphate-glucose	..	0.2 ml.	..	..	..
$HgCl_2$ (0.1M)	..	..	0.2 ml.	..	..
$HgCl_2$ (0.01M)	..	..	..	0.2 ml.	..
$HgCl_2$ (0.001M)	..	..	..	..	0.2 ml.
(*c*) Center well:					
KOH (10%) and filter strip	..	0.2 ml.	0.2 ml.	0.2 ml.	0.2 ml.

* Ringer phosphate-glucose is prepared as follows:

(*a*) Ringer solution: To 960 ml. of 0.154 *M* sodium chloride add 20 ml. of 0.154 *M* potassium chloride solution and 20 ml. of 0.11 *M* calcium chloride solution.

(*b*) Prepare *M*/15 phosphate buffer solution, *p*H 7.4, by mixing 80.4 ml. of *M*/15 sodium phosphate solution with 19.6 ml. of *M*/15 potassium dihydrogen phosphate solution.

(*c*) Mix 10 volumes of Ringer solution with 1 volume of the buffer solution.

(*d*) Add 2 ml. of 10 per cent glucose solution to every 100 ml. of the Ringer-phosphate solution.

Prepare a petri dish and a 100 ml. beaker with cold saline, resting in
larger beaker containing ice and water. Kill a rat by a sharp blow on t
head, quickly remove the liver and immerse in a dish of cold saline.* Cut
the largest lobe of the liver and place it on a filter paper tacked to a sm
board. Holding the tissue taut by means of a glass slide in the left han
slice a section of tissue at an angle of 45 degrees. Discard the first slice. Co

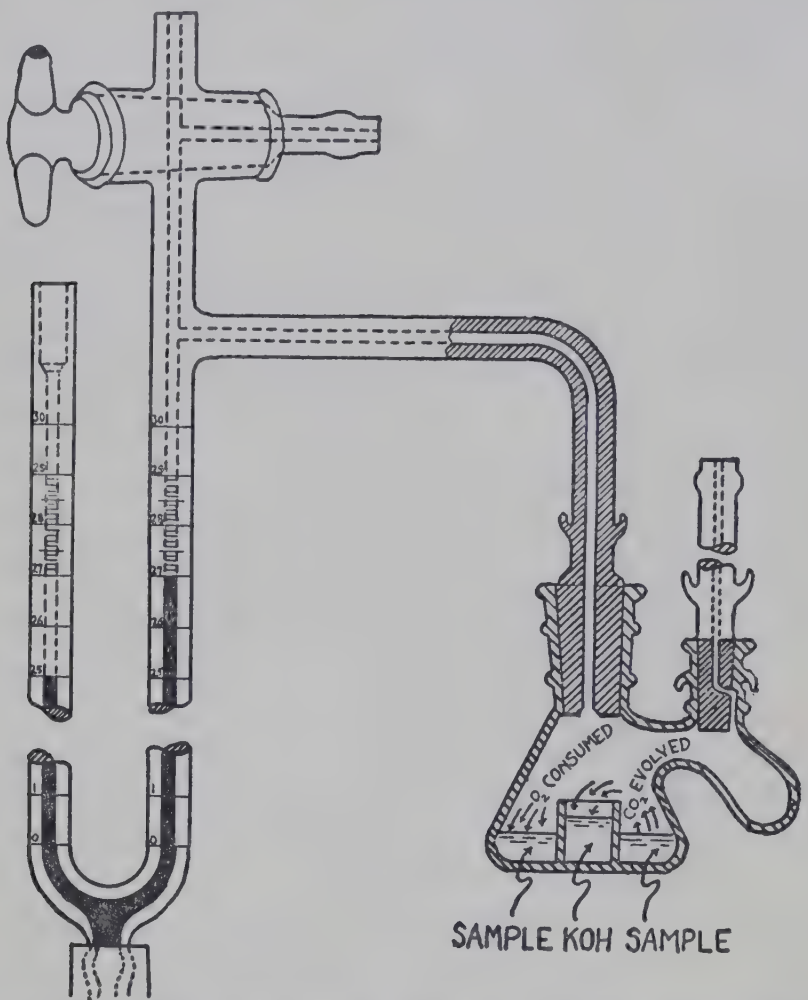


Fig. 9.—Warburg constant-volume manometer and reaction vessel. (Perkins, J. Ind. Eng
Chem. Anal. Ed., *15*.)

tinue slicing sections about 0.5 mm. thin. The cutting edge of the blad
should be visible through the slice. Transfer each slice to the beaker of col
saline. When a suitable number of slices have been cut (two to four pe
vessel), pass a stream of oxygen through the suspension of slices in salin
for one minute. Decant the supernatant and resuspend in cold saline. Remov
a slice onto a dry piece of filter paper and gently blot each side. Weigh it o
a suitable balance. When 100 to 200 mg. of wet weight of slices have bee
obtained, transfer these to a vessel. Do not put any tissue in vessel No. 1
which is the thermobarometer; it will measure any changes in temperature i
the bath and any changes in barometric pressure during the course of th
determination. Finally transfer several slices which have been weighed to

* The use of a Stadie slicer (A. H. Thomas) simplifies the procedure.

ared cup and place in an oven at 100°C. for a determination of its dry weight. Connect each vessel to its manometer and allow a stream of oxygen gas to flow through the manometer and vessel. Stopper each vessel and shut off its oxygen supply simultaneously at the end of five minutes. Remove each assembly to the bath and adjust the column of fluid in the manometers as each reacts to the increase in temperature by an expansion of the gas volume. Shake the manometers to equilibrate for ten minutes. Stop the shaker and adjust each manometer so that the right hand limb reads 150 mm. and read the height of the fluid in the left hand limb of the manometer. When the readings have been recorded, tip the contents of the side arms into the main compartment of each vessel. Continue shaking and take readings at regular intervals of fifteen minutes for as long as convenient, making the adjustment of the manometer fluid to 150 mm. each time before the reading is taken.

Correct each reading for temperature and pressure changes as indicated by the thermobarometer reading. Correct each value by multiplying by its vessel constant and plot the cubic millimeters of oxygen taken up against the time in minutes.

Calculate the Q_{O_2} for rat liver slices from your data, which include a knowledge of the dry weight of the tissue in each vessel. The Q_{O_2} is defined as the number of cubic millimeters of oxygen absorbed per milligram of dry tissue per hour.

(*a*) How do your values compare with those found in the references cited?

(*b*) What is the effect of Hg^{++}?

Chapter 5 **Saliva**

SALIVA is produced by the submaxillary, sublingual and parotid glands as well as by the mucous membrane and buccal glands of the mouth, throat a esophagus. Salivary flow is caused by mechanical, chemical or psych stimulation. The liquid contains, among other things, a protein (mucin), enzyme (ptyalin) and various inorganic ions.

The enzyme ptyalin has the power of hydrolyzing starch to maltose.

Collection of Saliva. After rinsing your mouth thoroughly with wate chew on a small piece of paraffin wax. This will stimulate the flow of saliv Transfer the accumulated saliva to a small beaker, and use for the followi experiments.

Experiment 1. Mucin. To 5 ml. of saliva in a test tube add 2 drops 0.1 *M* acetic acid. Use water as a control.

Remove the stringy precipitate, and perform the Millon test on th precipitate and the Benedict and Molisch tests on the saliva.

(*a*) What is the precipitate which forms?

(*b*) What function does this protein have as a component of saliva?

(*c*) What type of compound is mucin? How do your experimental result confirm this?

Experiment 2. Thiocyanates. To 5 ml. of saliva in a test tube add drops of 0.1 *M* ferric chloride and 1 drop of concentrated hydrochloric acid Use water as a control.

To eliminate the possibility that the red color may be due to ferri phosphate, add 5 drops of 1 per cent mercuric chloride solution (to form colorless mercuric thiocyanate). If the color is due to ferric phosphate, i will not disappear.

Experiment 3. Comparison of Activity of Dialyzed and Undialyze Saliva. Dialyze 10 ml. of saliva containing several drops of toluene agains running water for two hours. At the end of this time, use the dialyzed saliv for comparison with the undialyzed saliva in the experiment described below Add 5 drops of saliva to 25 ml. of a 1 per cent starch solution and mi thoroughly. At one minute intervals, transfer 1 ml. of the solution to a tes tube and add 2 drops of 0.01 *M* iodine. At the same one minute intervals transfer 1 ml. of the hydrolytic mixture to 5 ml. portions of Benedict's solu tion in labelled test tubes. (If the blue color with iodine solution still appear at the end of five minutes, add 10 drops of saliva to 25 ml. of the starch solution and begin again.)

Note the time when the achromic point (no color with iodine) is reached

Heat the tubes containing the Benedict solution for three minutes in

oiling water bath. Compare these results with those in the tubes to which odine solution had been added.

(*a*) What are the degradation products obtained when ptyalin acts on arch?

(*b*) How do the products in (*a*) behave towards iodine and Benedict's olution?

(*c*) Why is a positive Benedict test often obtained when the solution ives a red color with iodine solution?

(*d*) Does the dialyzed saliva hydrolyze the starch as rapidly as the ndialyzed? Why?

(*e*) What other method may be used to study starch hydrolysis?

Experiment 4. Influence of Temperature on Ptyalin Activity. Add ml. of a 1 per cent starch solution to each of four test tubes. Immerse one ube in an ice-water mixture, keep the second at room temperature, and place he third in a water bath at 38°C. Add to each of these tubes 2 drops of iluted saliva (1:9) and mix well. To the contents of the fourth tube add drops of the diluted saliva which had been heated in a boiling water bath or five minutes.

At intervals of five minutes remove a sample from each tube and test it with 2 drops of 0.01 *M* iodine as under Experiment 3. Note the rate of digestion in each tube.

(*a*) The contents of which tube reach the achromic point first?

(*b*) Devise a quantitative experiment to determine the effect of temperature on ptyalin activity.

Experiment 5. Estimation of Ptyalin. To 10 ml. of a 1 per cent starch olution in a test tube, add 2 ml. of 0.1 *M* sodium chloride, and place the tube n a water bath at 38°C. Prepare eight tubes so that each contains 3 ml. of vater and 3 drops of 0.01 *M* iodine. Now add 1 ml. of diluted saliva (1:9) o the tube containing the starch solution and mix the contents. At intervals of thirty seconds, add 2 drops of the digesting mixture to one of the tubes containing the iodine solution. Note the time when the addition of the digesion mixture to the iodine ceases to produce any change in color. If the time aken to reach this point (the "achromic point") is less than four minutes, further dilute the saliva with an equal volume of water and repeat the experiment.

(*a*) Assuming that 1 unit of amylase is the quantity required to digest 10 ml. of a 1 per cent starch solution to the achromic point in four minutes, calculate the number of units of amylase in 1 ml. of your saliva.

(*b*) Why is sodium chloride added to the starch solution?

Experiment 6. Determination of the *p*H Most Favorable to the Digestion of Starch by Saliva. Make up 10 ml. of each of the following buffer olutions (p. 149): *p*H 8, 7.4, 6.8, 6, 5.2. To each of these buffer solutions n a test tube, add 5 ml. of a 1 per cent starch solution, 2 ml. of 0.1 *M* odium chloride and 2 ml. of diluted saliva (1:9). Place all the tubes in a

water bath at 38°C. Determine in which tube the "achromic point" is f reached (consult Experiment 3). When this is obtained, add iodine so tion to each tube. (Tubes at *p*H 8 and 7.4 should be slightly acidified w acetic acid before the addition of iodine.)

(*a*) What is the optimum *p*H for ptyalin action?
(*b*) Why are tubes above *p*H 7 acidified before testing with iodine?
(*c*) How do you explain the continued action of ptyalin in the stomac

Experiment 7.* The Effect of Compounds which Inhibit or Destr Bacterial Activity on Salivary Amylase. Dilute 2 ml. of saliva with 8 m of water and mix well. Add 1 ml. of this diluted saliva to each of six te tubes. To each tube in turn add 5 drops of toluene, 5 drops of chlorofor 5 drops of 1 per cent mercuric chloride solution, 5 drops of 2 per cent phen solution, 0.5 gm. of sodium fluoride, and 5 drops of water. Let stand for t minutes and shake occasionally. Now add 5 ml. of a 1 per cent starch sol tion to each of the six tubes. Place all the tubes in a water bath at 38° for about fifteen minutes. At the end of this time, perform the iodine a Benedict tests on half of the contents of each test tube. Record your resul in tabular form.

(*a*) Do antiseptics and germicides as a rule inhibit the action of saliva amylase?

Experiment 8.* Some Inorganic Constituents of Teeth. Place tooth in 75 ml. of 1 *M* hydrochloric acid and allow to stand at room tempera ture for forty-eight hours. Shake occasionally. Note the evolution of ga Filter and separate the residue. Test a small portion of the residue and filtra for protein, using the appropriate tests (p. 18). Also, test the filtrate fo amino acids (Experiment 5, p. 19).

Add concentrated ammonium hydroxide to the filtrate until the reactio is alkaline. A precipitate should appear at this point. Dissolve the bulk of th precipitate by adding 30 per cent acetic acid solution while stirring. Test 5 m of the filtrate for phosphate ion by adding 2 ml. of ammonium molybda reagent and heating. To about 50 ml. of the filtrate add 25 ml. of 4 per cen ammonium oxalate solution. A precipitate should form. Warm the mixtu and let stand for fifteen minutes or longer. Filter through Whatman 42 pape (To the first few milliliters of clear filtrate, add a few drops of 0.2 *M* amm nium oxalate solution to test for complete precipitation. If a precipita forms, add an additional 5 ml. of ammonium oxalate.)

Transfer the filtrate to an Erlenmeyer flask, add 2 *N* ammonium hydroxid to alkalinity, stopper and let stand until the next period. A crystalline pr cipitate of ammonium magnesium phosphate forms. Examine some of th crystals under the microscope and draw a crystal.

(*a*) What gas is obtained when hydrochloric acid is added to a tooth
(*b*) What precipitate results upon the addition of ammonium hydroxid to the filtrate from the action of hydrochloric acid on a tooth?

* Adapted from Karshan, *A Laboratory Manual of Biochemistry for Dental Studen* (Columbia University, 1947).

(*c*) What precipitate is obtained at the point where ammonium oxalate added?

(*d*) List several inorganic and organic constituents of teeth.

Experiment 9.* Acid Production by the Action of Salivary Bacteria n Glucose. Collect about 6 ml. of unstimulated saliva (saliva collected th minimum movement of tongue, cheeks, lips and jaw) in a test tube and vide it into two equal parts. To one part add 0.4 ml. of 1 per cent glucose lution, and to another part add 0.4 ml. of water. Place both tubes in a water th maintained at 37°C. for three hours. At the end of this time, pipette ml. portions from each tube into small test tubes and add one drop of per cent phenolphthalein solution to each. Titrate with 0.01 *N* sodium droxide until a pink color appears that persists for about thirty seconds.

(*a*) Compare the results in the control (untreated saliva) with that in the liva—glucose mixture.

(*b*) Is there any evidence of acid production in (*a*)?

Experiment 10. The *p*H of Saliva. Test the reaction of saliva to omthymol blue, phenolphthalein and methyl orange solutions to obtain e approximate *p*H. Use *p*H papers also if available, such as Hydrion paper,† other suitable indicators.

For better results, the following procedure may be used. Prepare phosnate buffer standards of *p*H 6.2 to 7.6 (see p. 149). Use tubes of uniform iameter which fit into the comparator block. To each such tube containing) ml. of the phosphate buffer, add 0.5 ml. of 0.04 per cent of bromthymol lue solution. Mix thoroughly and stopper with a clean cork. These are the olor standards.

Add about 250 ml. of distilled water to a flask, and adjust to a *p*H of 6.8 7 by the careful addition of 0.01 *N* sodium hydroxide, using bromthymol lue as an outside indicator. (If too much sodium hydroxide has been added, educe the *p*H of the water with 0.002 *N* hydrochloric acid, adding about 5 rops at a time.)

To a dry test tube which fits into the comparator block, add 0.5 ml. of .04 per cent bromthymol blue solution, 9 ml. of the adjusted water of *p*H .8 to 7, and 1 ml. of saliva. Stir and compare with the color standards in a omparator block. To compensate for the turbidity of the salvia, a tube conaining water is placed behind the tube containing saliva and indicator, and tube containing 9 ml. of water plus 1 ml. of saliva is inserted behind the olor standard.

The *p*H of saliva may also be determined by means of a glass electrode H meter (see p. 34).

(To avoid loss of carbon dioxide, collect the saliva under oil as follows: dd about 5 ml. of paraffin oil to a clean test tube. Insert one end of a glass ube of about ½ inch diameter into your mouth and allow the saliva to accuulate under the layer of oil. Avoid blowing through the tube.)

* From Karshan, *A Laboratory Manual of Biochemistry for Dental Students* (Columbia niversity, 1947).

† Purchased from Machlett and Sons, New York City.

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Chapter 6 **Milk**

Milk is almost a perfect food, containing proteins, fats, lactose, inorga salts and vitamins. It is a dilute food, however, owing to its high wa content, and it is low in iron and vitamin C. Casein is its chief protein. phosphorus content is high, and the element is present in two forms: lin in the casein molecule and as inorganic phosphate ion.

The separation of casein from milk depends on its precipitation from a solution, dragging down with it the milk fat. The latter is extracted with organic solvent in which casein is insoluble. The filtrate from the casein a fat contains the soluble constituents such as lactose and inorganic phosphat and other ions.

Casein may be separated from milk by the clotting action of rennin in presence of calcium ion (pepsin, too, will clot it to some extent), converti the soluble calcium salt of casein into insoluble calcium paracaseinate.

Experiment 1. Dialysis of Milk. Pour 50 ml. of milk into a dialyzi bag (p. 24) and, leaving some empty space, tie the upper part of the b with a string. Suspend the bag in a 400 ml. beaker by tying the string tached to the bag over a glass rod placed horizontally over the beaker. not allow the bag to touch the bottom of the beaker. Add distilled water the beaker until it is level with the upper surface of the milk in the s Allow to dialyze for one and one-half hours. Then test the dialysate f protein (p. 18), reducing sugar (p. 2), chloride ion, phosphate ion a calcium ion.

Explain the results.

Experiment 2. Preparation of Casein. Warm 100 ml. of milk in hot water bath until the temperature of the milk reaches 40°C. Add dropwi with stirring about 1 ml. glacial acetic acid until all of the casein flocculat Filter the coagulum through cheese cloth and gently squeeze most of t water from the casein. Suspend the precipitate, by rubbing, in 50 ml. of per cent ethanol. Decant and discard the supernatant. Repeat using 50 m of a 1:1 ether-ethanol mixture. Use another 50 ml. of the ether-alcohol transfer the casein to a Buchner funnel and wash with 50 ml. ether. Su the precipitate dry and remove the last traces of ether by spreading on watch glass.

Experiment 3. Color Tests on Casein. Perform the Millon, biu and Hopkins-Cole tests, using small quantities of the solid casein prepar in Experiment 2. (Refer to p. 18 for tests.)

(*a*) Can positive tests be obtained, using solid proteins?

(*b*) Why is casein considered a "biologically wholesome" protein?

Experiment 4. Milk Fat. Evaporate the ethereal extract from Exper ment 2 on the steam bath. **(Caution! Flames!)** Note the odor of the residu

Devise experiments to prove that the residue is a fat. (Consult p. 12, Experiment 3, etc.)

(*a*) What are some of the common food products prepared from milk?

Experiment 5. Effect of Rennin on Milk. Place two test tubes each containing 5 ml. of fresh milk in a water bath at 38°C. for five minutes. To one test tube, add 1 ml. of 0.1 per cent rennin suspension, and to the other, 1 ml. of 0.1 per cent pepsin suspension. Shake and replace in the water bath at 38°C. Note the time at which each coagulates.

(*a*) Explain why the pepsin preparation is also effective as a clotting agent.

(*b*) Do you note any difference in action between the pepsin and the rennin?

(*c*) What is an industrial application of this reaction?

Experiment 6. The Effect of Calcium on the Clotting of Milk. Add 1 ml. of 0.2 *M* ammonium oxalate to 10 ml. of milk. Mix well. Prepare two test tubes as follows:

.....	5 ml. of oxalated milk	2 ml. of 0.5 *M* $CaCl_2$	1 ml. of 1 per cent rennin
.....	5 ml. of oxalated milk	2 ml. of water	1 ml. of 1 per cent rennin

Place the tubes in a water bath at 38°C. for ten minutes. Note the result.

(*a*) Is there a physiologic need for the clotting of milk?

Experiment 7. Isoelectric Point of Casein. Place 0.3 gm. of casein (Hammarsten's) into a 50 ml. volumetric flask, and add 25 ml. of water, previously warmed to 38°C., and 5 ml. of *M* sodium hydroxide. Shake gently to dissolve the casein. Add 5 ml. of *M* acetic acid, mix, cool, and make up to 50 ml. with water. Filter, if necessary. The solution (casein in 0.1 *M* sodium acetate) should be faintly opalescent. Using pipettes, carefully make up the following mixtures (use dry test tubes). (For fractions of a milliliter use the 1 ml. Mohr pipette graduated in hundredths.) Allow to stand for thirty minutes. Note the results.

Test tube.....	1	2	3	4	5	6	7	8	9
Ml. of casein solution.....	1	1	1	1	1	1	1	1	1
Ml. of water.....	8.4	7.8	8.8	8.5	8	7	5	1	7.4
Ml. of 0.01 *M* acetic acid.....	0.6	1.3							
Ml. of 0.1 *M* acetic acid.....	..	..	0.3	0.5	1	2	4	8	
Ml. of *M* acetic acid.	..	..	..	..	..	..	..	..	1.6
Approximate *p*H*...	5.9	5.7	5.3	5	4.7	4.4	4.1	3.8	3.5

* If a *p*H meter is available, check the *p*H of each tube.

(*a*) How does the measured *p*H compare with the calculated?

(*b*) What is the *p*H of the tube showing greatest turbidity?

(*c*) How does your determination of the isoelectric point compare w that reported in the literature?

(*d*) What property of the protein at the isoelectric point does this exp ment illustrate?

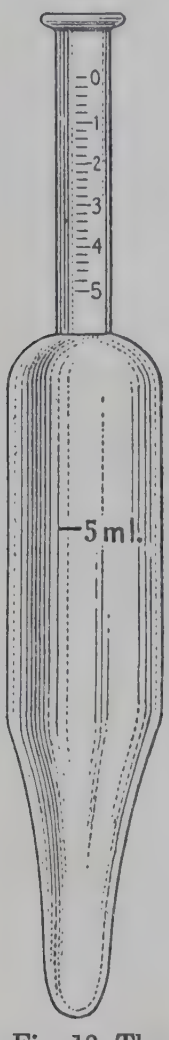

Fig. 10. The Babcock tube.

Experiment 8. The Determination of Fat by the Ba cock Method. The fat content of milk is convenien determined by the Babcock method, which depends upon relative stability of fats (compared to other milk consti ents) in strong acid. After mixing milk with concentrat sulfuric acid the fat is separated by centrifuging and amount is determined by the volume it occupies in the s cially calibrated neck of the Babcock tube (Fig. 10). accurate measurements of milk fat are needed, see the v ume published by the Association of Official Agricultu Chemists.*)

By means of a medicine dropper whose tip is drawn to long capillary, fill a Babcock tube with the milk up to t 5 ml. mark. With a similar pipette add concentrated sulfu acid (specific gravity, 1.82) slowly while shaking, until t volume reaches the calibrated neck of the tube. Do not all the contents to become hot. Shake the tube until all trac of the curd disappear. Fill the neck of the tube up to t zero mark with a 1:1 solution of concentrated hydrochlo acid and amyl alcohol. Centrifuge the tube for five minut If the top of the yellow fat column is not at the zero ma add warm water to bring it there and centrifuge again f two minutes. The percentage of fat is read from the positi of the lower meniscus of the fat column.

The fat should be translucent, golden-yellow and free fro particles. If it is milky or charred, the determination shou be repeated, taking special precautions not to overhe locally by insufficient mixing of the acid and the mil Should the fat column reach beyond the calibration, repe the determination on a 4:1 or 3:2 dilution of milk and water. If cream is to analyzed, one volume of it is first diluted with four of water, and aft thorough mixing, the determination is performed as on milk. In this ca the percentage of fat reading is, of course, multiplied by five.†

* A.O.A.C., 6th edition, page 311 (1945).

† A modification of this method does away with the use of sulfuric acid and wi centrifugation (Schain, Science, *110*:121, 1949).

Chapter 7 Gastric Digestion

COMMERCIAL pepsin is a crude enzyme preparation obtained from the gastric mucosa of cattle. It usually consists of a mixture of pepsin and rennin, the latter being a milk-clotting factor.

The products of peptic digestion are an ill-defined mixture of partially split proteins called metaproteins, proteoses, peptones and polypeptides. They differ in molecular weight.

There are numerous methods for studying quantitatively the optimum conditions (*p*H, temperature and so on) for peptic activity. The most accurate would be to follow the increase in free amino groups as digestion proceeds, by means of the amino nitrogen determination (see p. 32). In Experiments 2 to 4 the substrate* is a coagulated and insoluble protein with a dye (iodoeosin) adsorbed onto it. When pepsin digests this protein it dissolves the protein during its initial stage of enzyme action, thus liberating the dye and coloring the solution.

For the following experiments the student will prepare 100 ml. of a 0.4 per cent filtered pepsin solution in water. Set up a large beaker of water kept at 40°C. for incubating the digestion mixtures.

Experiment 1. Conditions Essential for the Action of Pepsin. Prepare the following test tubes as indicated:

Tube	*Pepsin*	*0.2 M HCl*	*Water*	*0.2 M $HgCl_2$*
.....	1.5 ml.	..	1.5 ml.	
.....	..	1.5 ml.	1.5 ml.	
.....	1.5 ml.	1.5 ml.		
.....	1.5 ml.	1.5 ml.	..	2 drops

To each tube add a pinch of albumin-iodoeosin (see Appendix) and shake. Incubate for one hour at 40°C. Shake occasionally during the course of the digestion. Allow the particles to settle and observe the clear supernatant fluid.

(*a*) In which tube has digestion taken place?
(*b*) What is the nature of the action of mercuric chloride?
(*c*) What is the purpose of tube 2?

Experiment 2. Effect of Temperature on Peptic Activity. To each

* Another substrate which may be used, but which requires more time for evidence of digestion, is the Mett tube.

Cut several glass tubes of 2 mm. diameter into ten inch lengths. Draw egg white into the tubes and seal each end in a flame. Immerse the tubes in a boiling water bath until the egg white is thoroughly coagulated.

Cool to room temperature, cut off the ends, and cut the remaining tube into ¾ inch lengths by means of a file.

of four tubes add 1.5 ml. of the pepsin solution and 1.5 ml. of 0.2 *M* hydr chloric acid.

1. Place in a beaker of ice-water.
2. Place at room temperature.
3. Place in the bath at 40°C.
4. Boil for one minute. Cool to room temperature and place in the bath 40°C.

To each tube add a pinch of the albumin-iodoeosin and incubate as Experiment 2. Observe the filtrates for evidence of digestion.

5. Take tube (1) and place it in the bath at 40°C. and incubate for o hour. Observe the filtrate.

(*a*) Of tubes 1 to 4, which shows best digestion?
(*b*) What is the effect on an enzyme of low and high temperature Is either reversible?

Experiment 3. Effect of *p*H on Peptic Activity. Prepare four tub as follows:

Tube	*Pepsin*	*0.2 M HCl*	*Water*	*Buffer pH 6*	*Buffer pH 7*	*pH of soluti*
1.....	1.5 ml.	1.5 ml.	..	..	..	1.5
2.....	1.5 ml.	4 drops	1.5 ml.	..	..	2.5
3.....	1.5 ml.	..	..	1.5 ml.	..	6
4.....	1.5 ml.	..	..	..	1.5 ml.	7

Add a pinch of albumin-iodoeosin to each tube and incubate for one hou Observe the supernatants.

(*a*) What is the optimum *p*H for peptic digestion?
(*b*) Will the nature of the acid used affect the digestion at the same *p*H

Experiment 4. Determination of Gastric Acidity. Hydrochloric aci is present in both free and "combined" form in the stomach. The relativ quantity of each is of diagnostic value. Samples of gastric juice are obtaine from fasting subjects after the stimulation of gastric secretion by the feed ing of test meals or by the injection of histamine. The gastric juice i strained and the determination is performed on the undiluted, partiall clarified fluid.

Pipette 5 ml. of strained gastric fluid into a porcelain evaporating dis and dilute with 10 ml. of distilled water.

Add 2 drops of Töpfer's reagent (a 0.5 per cent solution of p-dimethyl amino-azobenzene in 95 per cent alcohol) and titrate with 0.1 *N* NaOH to yellow color (*p*H 4). The volume of alkali required represents the free HC Now add 2 drops of phenolphthalein and continue the titration with th alkali until the solution turns pink (*p*H 8.5). The second volume of alkal represents the "combined acid." If a *p*H meter is available the determina tion can be conveniently performed by titrating first to p*H* 4 and recordin the volume of alkali and then continuing the titration to *p*H 8.5.

Note: If gastric juice is not available it can be simulated by mixin

arious ratios of 0.2 *M* acetic acid and 0.2 *M* HCl to serve as "unknowns." A pinch of casein, added to the acid solution, can be used to provide the olid matter for the artifact.

(*a*) Are any organic acids titrated when alkali is added up to *p*H 4?
(*b*) Calculate the percentage of 0.1 *N* HCl which is not titrated at *p*H4.

Chapter 8 **Pancreatic Digestion**

PANCREATIC juice contains trypsin, a proteolytic enzyme; pancreatic amyl (amylopsin), an amylase similar in action to ptyalin; and pancreatic lipa a fat-splitting enzyme. Pancreatin is the dried and partially purified extr of the pancreas.

Experiment 1. Pancreatic Digest. One week before Experiments 2 4 are to be performed, prepare a pancreatic digest. Dissolve 0.25 gm. pancreatin or any other commercial trypsin preparation* in 50 ml. of wat Neutralize to litmus, using 0.05 *M* sodium carbonate. Add, a little at a tin 5 gm. of casein, and stir well. Add slowly, with stirring, 50 ml. of 0.1 sodium carbonate. Preserve with toluol and incubate for a week at 38°C. in stoppered flask.

Experiment 2. Products of Pancreatic Digestion. Immerse the inc bated pancreatic digest from Experiment 1 in a boiling water bath, and aft ten minutes add 0.2 *M* acetic acid, dropwise, until the mixture is acid litmus. Cool. Filter. Use the filtrate for Experiment 3.

(*a*) What is the precipitate?

Experiment 3. Test for Tryptophan. To 5 ml. of filtrate add bromi water, drop by drop, until a pink color develops. Run a control, using 5 m of water.

To another 5 ml. of the filtrate add 10 drops of dilute sulfuric acid (1:2 and 10 ml. of a 10 per cent solution of mercuric sulfate in *M* sulfuric aci Shake. Allow to stand for ten minutes. Filter. Use the filtrate for Exper ment 4.

(*a*) What is the precipitate?

Experiment 4. Test for Tyrosine. Perform the Hopkins-Cole (p. 19 the Millon (p. 18) and the xanthoproteic (p. 18) tests on samples of th filtrate.

(*a*) Does mercuric sulfate solution separate tryptophan from tyrosine?
(*b*) What amino acids are present in the filtrate?

For the following experiments the student will prepare an extract of commercial powder. The dilution of the enzyme will depend on its activit

* An excellent source of trypsin may be obtained from the Viobin Corp., Monticell Ill. The powder represents the whole pancreas tissue, de-fatted and dried. It is stable a may be extracted with water and filtered to obtain the trypsin in solution. The extra also contains pancreatic amylase and lipase used in Experiments 9 and 10.

or the Viobin Pancreas powder (see footnote, p. 58), extract 0.25 gm. of ıe powder with 100 ml. of water and centrifuge. Pipette off the clear ıpernatant and use for Experiments 5 to 8.

The substrate used to test for tryptic activity in the following experiments ; the layer of gelatin found on photographic negatives. For this purpose, xpose to the sunlight a roll of film, develop, fix and wash it. The dried, paque negative is cut into strips about ½ by ¼ inch. When trypsin digests ıe gelatin of the film strip, the reduced silver (black) on the cellulose film ·ill fall off and leave a transparent film. In those cases where digestion has ot taken place, the film strip remains black.

Experiment 5. Effect of Enzyme Concentration on Digestion Rate. 'repare a large beaker of water maintained at 37°C. Prepare eight tubes as irected:

Tube	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
uffer *p*H 8.0	3	3	3	3	3	3	3	3
rypsin	3	2	1	0.5	0.4	0.3	0.2	0
Vater	0	1	2	2.5	2.6	2.7	2.8	3

Place all tubes in the water bath at 37°C., and at "zero" time drop one ilm strip into each tube, making certain that the strip is immersed in the olution. Gently rotate each tube, and when the first two or three tubes show :lear strips, quickly add some formalin to each tube and remove from the ›ath. Decant the formalin and replace with about 5 ml. of fresh formalin. After ten minutes decant the formalin and wash with several changes of water. The strips may be placed emulsion side up on a sheet of filter paper for ›bservation, or permanently recorded by means of transparent Scotch tape n your note book.

An alternate measurement in this experiment may be to note the time aken to clear the film in each tube.

(*a*) What is the purpose of tube (8)?
(*b*) Define your own unit of trypsin activity.
(*c*) What is the purpose of the formalin?

Experiment 6. Effect of *p*H on Tryptic Digestion. Prepare the following tubes. Into each tube place 3 ml. (or an amount found in Experiment 5 to digest in a convenient time, e.g., fifteen min.) of the trypsin solution. Into each tube then place 3 ml. of the appropriate buffer solution.

Tube	*pH of Buffer*
1	5.4
2	6
3	7
4	8

Place in the water bath at 37°C., and at "zero" time add a strip of film to ;ach. When the first tube shows definite signs of digestion, add formalin to ıll and compare.

(*a*) What is the optimum *p*H for tryptic digestion?

(*b*) Would any of the other tubes show activity if allowed to incuba longer? Why?

Experiment 7. Effect of Inhibitors on Tryptic Activity. Prepare t following tubes:

Tube	*Trypsin*	*Buffer pH 8*	*Water*	*0.2 M HgCl*
1.....	3 ml.	3 ml.	..	..
2.....	3 ml.	3 ml.	..	2 drops
3.....	..	3 ml.	3 ml.	..

Repeat as for Experiment 5.

(*a*) What is the nature of the action of mercuric chloride on trypsin?

(*b*) What other inhibitors might you try?

Experiment 8. Effect of Temperature on Tryptic Activity. Prepa three test tubes containing 3 ml. of buffer, *p*H 8, and 3 ml. of trypsin solu tion. Then

1. Incubate at 37°C.
2. Boil for one minute, cool to room temperature, and incubate at 37°C
3. Place in an ice-water bath.

Add a film strip to each tube and allow to incubate until the first tub shows digestion. Place tube (3) at 37°C. and allow it to incubate for th same length of time.

(*a*) What temperature is optimum?

(*b*) Is the effect of low temperature on the enzyme reversible?

Experiment 9. Pancreatic Amylase. Prepare a 0.5 per cent extract o a pancreas powder, using 0.05 *M* sodium carbonate for the extraction. Pre pare several test tubes as follows:

Tube	*Pancreatic Extract*	*1 Per Cent Starch*
1.....	2 ml.	5 ml.
2.....	2 ml. boiled for one minute and cooled	5 ml.
3.....	2 ml. of water	5 ml.

Incubate at 37°C. for thirty minutes. Cool and divide each solution into two parts. Test one with iodine solution and the other with Benedict's reagent.

(*a*) Which tubes give a positive iodine test? Why?

(*b*) Which tubes give a positive Benedict's test? Why?

(*c*) Which sugar is formed?

Experiment 10. Pancreatic Lipase. FAT EMULSION. Add 1 ml. of olive oil to 5 ml. of alcohol. Mix. Add an equal volume of water and shake thoroughly. Add 10 drops of phenol red and enough 0.1 *M* sodium carbonate to make the mixture definitely pink. PANCREATIC EXTRACT. Neutralize

me of the pancreatic extract prepared for Experiment 9, so that it is finitely pink to phenol red. Set up the following tubes:

	(1)	*(2)*
Fat emulsion	6 ml.	6 ml.
Pancreatic extract	3 ml.	3 ml. boiled for 1 minute and cooled

cubate for twenty minutes at 37°C. and observe the color of the indicator. place of the fat emulsion, sweet cream may be used.

(*a*) Explain the results. What is the *p*H range for phenol red?
(*b*) How could you make this experiment a quantitative one?

Chapter 9 **Bile**

SOME of the more important constituents of bile are the bile acids, bile p ments and inorganic ions. The bile acids exist mostly in the form of pepti linkages of glycine and taurine with cholic acid, desoxycholic acid a lithocholic acid.

Experiment 1. Influence of Bile on the Action of Lipase. Prepa the following mixtures:

	(1)	*(2)*	*(3)*
Pancreatic extract (p. 58, footnote)	2 ml.	2 ml.	2 ml. boiled for o minute, cooled
Fat emulsion (p. 60)	5 ml.	5 ml.	5 ml.
Bile (neutralized)	..	1 ml.	1 ml.
Water	1 ml.	..	...

Shake each thoroughly and incubate in a beaker of water kept at 37°C. f one hour. Shake each tube at fifteen minute intervals. Wash the contents each tube into a separate labeled beaker with 20 ml. of neutralized 95 p cent alcohol. Add 5 drops of phenolphthalein and titrate with 0.05 *N* sodiu hydroxide to a definite pink color. Record your results.

(*a*) Which tube required the most alkali for neutralization? Why?
(*b*) What constituent of bile is responsible for this effect? Why?
(*c*) What is the effect of boiling on the lipase?

Experiment 2. Test for Bile Pigments (Gmelin's Test). Add 3 m of concentrated nitric acid to a test tube, and by means of a pipette carefull place on the surface of this an equal amount of bile. Shake the tube gently

(*a*) What causes the play of colors?
(*b*) What is the origin of the bile pigments?
(*c*) What are some of the interrelationships of the pigments of the body

Experiment 3. Test for Bile Pigments (Van den Bergh Test). Ad 5 ml. of diluted bile to 2 ml. of freshly prepared Ehrlich's diazo reagent Repeat with water instead of bile.

(*a*) What do you observe?
(*b*) What are the two important bile pigments?
(*c*) In what form are they excreted in the urine and feces?

Experiment 4. Test for Bile Acids. To 3 ml. of bile diluted 1:5 ad a crystal of cane sugar and shake until it dissolves. Slowly add 3 ml. of con

ntrated sulfuric acid, inclining the tube so that the acid settles to the ottom. Gently rotate the tube. What is the result?

(*a*) What is the chemistry of this reaction?
(*b*) Why cannot this test be applied to urine?

Experiment 5. Effect on Surface Tension. To illustrate the property lowering surface tension which the bile salts have, perform the following st: Pour 5 ml. of bile into a test tube. Sprinkle some powdered sulfur on the rface. Repeat the test, using water instead of bile. What is the difference? What ions are present in bile?

Experiment 6. Isolation of Bile Salts. Stir 25 ml. of ox bile with colorizing carbon to give a paste. Place in an evaporating dish on the steam ath until a dry mass is obtained. Grind to a powder in a mortar and boil ith 50 ml. of absolute alcohol on the steam bath for twenty minutes. Filter ot through a fluted filter paper. To the filtrate, cooled to room tempera- re, add ether with mixing until the mixture is cloudy. Allow to stand until ystals form. Examine under the microscope. Perform the test for bile acids nder Experiment 4 and try the effect of a small amount in place of whole ile in Experiment 1.

Chapter 10 **Blood**

A NUMBER of chemical tests for blood—which, incidentally, do not dif entiate human from other bloods—are given. These include the import hemin test, and the method of detecting blood in a blood stain. Tests fo number of constituents are also included.

Experiment 1. Hemin Test. Spread a drop of blood (to form a t film) on a glass slide. Pass the slide back and forth over a small fla Slowly evaporate until the blood is quite dry. Be careful not to burn blood when drying. To the film add a drop of *M*/75 potassium chloride glacial acetic acid. Cover with a slip and heat cautiously over a small fla until bubbles appear and active boiling takes place. Immediately rem from the flame and add another drop of the reagent under the slip. Allow cool and examine under the microscope. Make a drawing of the crystal

(*a*) Explain the formation of hemin.

Experiment 2. Preparation of Hemin. Heat 150 ml. of glacial ace acid and 5 ml. of saturated sodium chloride to 100°C. in a 2 liter flask. (U sand bath under the hood.)

During the course of twenty to thirty minutes, 55 ml. of blood (fi filtered through linen cloth, if necessary), are dropped from a separato funnel into the hot solvent, without interrupting the heating. (The blood not allowed to come in contact with the walls of the flask as it flows in

After the blood has been added, continue boiling the liquid gently f fifteen minutes. Most of the hemin separates out.

The mixture is cooled to 40° to 50°C. and filtered at this temperatu

The hemin is washed successively with 50 per cent acetic acid, wat alcohol and ether (25 ml. each). Dry. Examine under a microscope.

Hand in the hemin to the instructor.

Experiment 3. The Benzidine Test. Set up test tubes each containi 2 ml. of blood diluted as follows: 1:200, 1:400, etc., until a negative test obtained. To each tube add 3 ml. of a freshly prepared saturated solution benzidine in glacial acetic acid, and 1 ml. of 3 per cent hydrogen peroxid Shake each tube well. Note the color developed.

(*a*) What is the mechanism of this reaction?

(*b*) What is the greatest dilution at which the color change is still visib according to your experiment?

Experiment 4. Tests for Constituents. Add 50 ml. of water to 10 m of blood in an evaporating dish and heat on a steam bath. Add two drops

* Sodium hydroxide solution is effective in loosening dried blood from glass.

2 *M* acetic acid and continue heating for five minutes, until a coagulum forms and the supernatant liquid is clear. Filter. Reserve the precipitate (label *A*). Evaporate the filtrate to one-half its volume, filter, if necessary, and perform the following tests on the filtrate: Benedict's, chloride, and phosphate.

Incinerate one-fifth of the coagulum (*A*) in a porcelain crucible (under the hood). Cool. Dissolve in 3 to 5 ml. of *M* hydrochloric acid and add 1 ml. of 0.2 *M* potassium ferrocyanide.

(*a*) Prepare a list of the various constituents of blood.

Experiment 5. Fibrin. Perform Millon's (p. 18), Hopkins-Cole (p. 19), and biuret (p. 19) tests on a small piece of fibrin. Result?

(*a*) What is the relationship of fibrin to fibrinogen?

Experiment 6. Detection of Blood Stains. Take a sample of the stained cloth, add 3 ml. of 0.1 *M* sodium hydroxide, allow to stand for three minutes, neutralize (to litmus) with 0.2 *M* acetic acid, remove the cloth and concentrate to dryness on the steam bath. Scrape the residue together and treat a small amount on the slide as described under Experiment 1 (Hemin test).

Experiment 7. Spectroscopic Examination of Blood. Set up a series of six test tubes containing fresh, defibrinated blood diluted as follows: 1:50, 1:100, 1:200, 1:300, 1:400 and 1:500. Each solution should be filtered or centrifuged if not clear. Examine spectroscopically in a direct vision or angular vision spectroscope.

The absorption bands are due to oxyhemoglobin. Make a drawing of the spectrum showing the position of the absorption bands.

Add a few drops of freshly prepared ammonium sulfide solution or, even better, Stokes' reagent to each of the solutions. Without shaking, examine immediately in the spectroscope. Compare the absorption spectrum of reduced hemoglobin with that of oxyhemoglobin.

To 5 ml. of diluted blood (1:20) add 2 drops of a freshly prepared 10 per cent solution of potassium ferricyanide. Note the color of the mixture. Examine spectroscopically. Dilute cautiously until the furthest band to the left is most prominent. This is the absorption spectrum of neutral methemoglobin.

(*a*) Why is it desirable to make the spectroscopic examination of the tubes containing reduced hemoglobin immediately, and also to avoid shaking?

(*b*) How is human blood distinguished from animal blood?

(*c*) Give the names of four blood proteins and mention one specific function of each.

(*d*) What is the approximate molecular weight of hemoglobin, and how was this determined?

(*e*) What is the percentage of iron in hemoglobin?

(*f*) Describe a theory of blood coagulation.

Chapter 11 **Colorimetry**

COLORIMETRIC methods for quantitative chemical determinations are base on the possibility of converting the constituent to be estimated into a su stance whose solution (or suspension) is highly colored. Many substanc will react with suitable reagents to form soluble end products of such inten coloration that micro quantities may be determined.

All colored solutions absorb some light. Lambert investigated the relatio ship between the intensity of the incident and the transmitted light, in term of the thickness of the layer of solution through which it passes. His co clusions—Lambert's law—can be summarized as follows:

$$\log_{10} \frac{I_0}{I} = k't$$

I_0 = Intensity of incident light
I = Intensity of transmitted light
t = Thickness of the layer of solution traversed by the light
k' = A constant whose value depends on such factors as units, solvent, temperature, wavelength, and so on.

Beer studied the relation between the concentration of the colored solutio and the amount of monochromatic light absorbed by it. His law can b formulated thus:

$$\log_{10} \frac{I_0}{I} = k''c$$

c = The concentration
k'' = A constant whose value depends upon such factors as those mentioned above

By combining the two laws, the Lambert-Beer relationship may be applie to simultaneous variations of t and c.

$$\log_{10} \frac{I_0}{I} = kct$$

Solutions of simple colored substances have been found to obey the Lambert-Beer law rigidly if the conditions are adhered to. However, in prac tice, the source of light is seldom monochromatic, and the law is not always strictly applicable. Also, changes in light absorption may occur when asso ciation, dissociation or a shift in equilibrium accompanies dilution of the solution. The law does not take such factors into consideration.

A variety of instruments have been devised for measuring or matching the colors of solutions. By applying the Lambert-Beer relationship to such measurements a quantitative determination may be performed.

VISUAL COLORIMETRY

The Duboscq colorimeter was designed for visual matching of colors, using a white or incandescent tungsten light source. Since it is a color comparator, it is necessary, in performing quantitative estimations with it, to run a standard solution of known concentration along with the unknown. Then, if both samples are treated identically, the final colored solutions may be matched by varying the thickness of the layers traversed by light of the same intensity. Under these conditions the Lambert-Beer law gives

$$\log_{10} \frac{I_0}{I} = kc_1t_1 \text{ and } \log_{10} \frac{I_0}{I} = kc_2t_2$$

Since I_0, I and k are the same in both cases,

$$c_1t_1 = c_2t_2$$

$$\text{or } \frac{c_1}{c_2} = \frac{t_2}{t_1}$$

This formula is usually expanded to make it more convenient for the calculation of blood constituents.

$$C_u = \frac{R_s}{R_u} \times m_s \times \frac{100}{v} \times \frac{D_u}{D_s}$$

where C_u = milligrams of unknown per 100 ml. of blood or "milligram per cent"
m_s = number of milligrams of the constituent in the standard solution
R_u = reading of the unknown cup
R_s = reading of the standard cup
v = volume of blood actually used in the color production
D_u = volume to which the unknown is diluted
D_s = volume to which the standard is diluted

EXAMPLE. For details of the methods used in this example, see Experiment 1 (Preparation of a Protein-Free Blood Filtrate, p. 79) and Experiment 8 (Determination of Creatinine, p. 95).

m_s = 0.030 mg. (5 × 0.0060 mg.)
v = 1.00 (10.00 ml. of filtrate = 1.00 ml. blood)
R_s = 15.0
R_u = 17.3 av.
D_u = 15 (10.00 + 5.00)
D_s = 30 (5.00 + 15.00 + 10.00)

$$C_u = \frac{15.0}{17.3} \times 0.030 \times \frac{100}{1.00} \times \frac{15}{30} = 1.3 \text{ mg. per 100 ml.}$$

The operation of a colorimeter is as follows:

Light from a source of uniform intensity is transmitted in parallel rays by the mirror (*I*) (Fig. 11) through the two fluids to be compared. These solutions are contained in metal-jacketed, thick-walled, cylindrical cups (*H*). The bottom of each cup is formed by a plate of clear glass. The cups are supported on a movable platform which may be raised or lowered by a rack and pinion. Two fixed, clear glass plungers (*G*) enter the liquids as the cups are

raised. The distance between the lower surface of the plunger and the bottom of the cup may be read from a millimeter scale equipped with a vernier on the side of the instrument. Thus the thickness of the layers of the colored liquids through which the light passes is obtained.

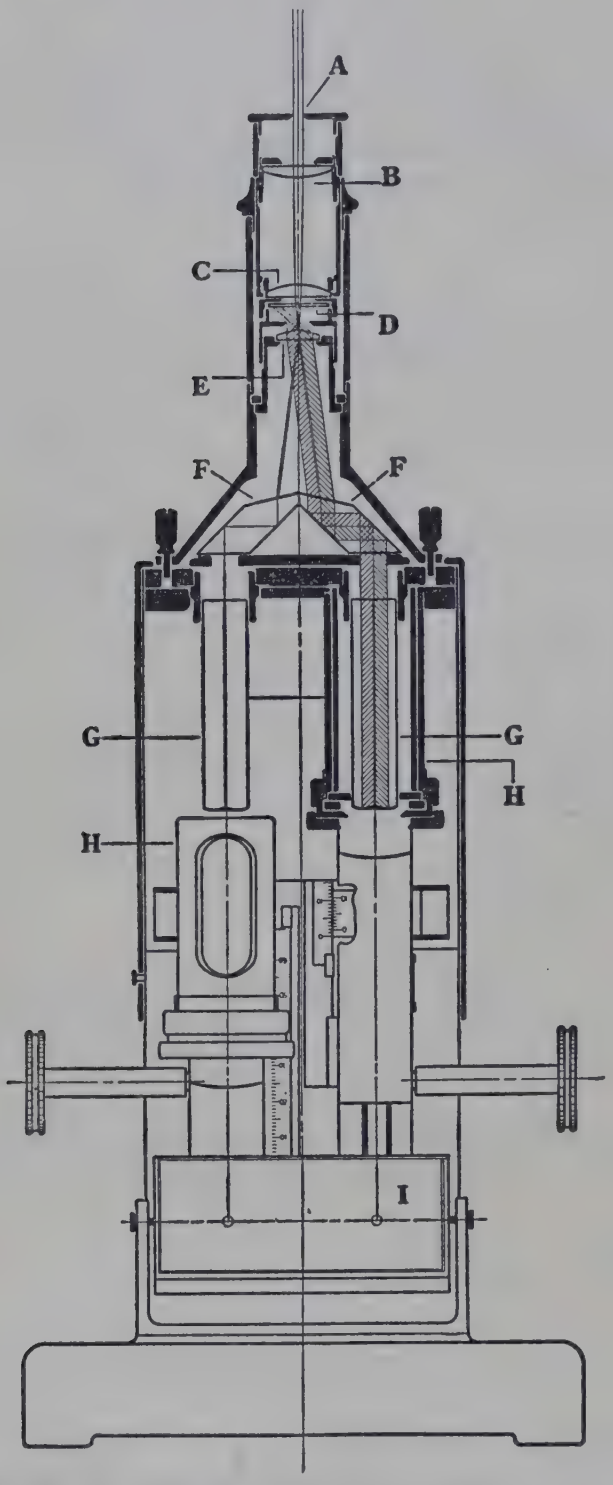

Fig. 11. Sectional view of colorimeter. (Bausch & Lomb Optical Co.)

The light enters the plungers, and is reflected by a pair of prisms (*F*) through several lenses (*E*, *D*, *C* and *B*) and to the eye. The circular field appears split in two, each half of which is due to the light transmitted through the liquid in each of the two cups.

Since the depth of one or both columns of liquid may be varied, they are adjusted that the intensity of colors of the two solutions appears equal the field.

A number of points should be kept in mind in the use of the instrument:

1. Care must be taken that the source of light is such that both fields ill be equally illuminated. Special colorimeter illuminators may be secured or this purpose. Extraneous light leaks may be minimized by using the shield ith which the instrument is equipped. One may test for evenness of illumiation by filling both cups with the same colored solution, setting both cales at the same reading, and noting whether the two fields match. If they o not, sometimes a slight shift in the angle which the colorimeter makes to ne light source will correct the difficulty. (Uneven lighting is frequently aused by injury to the mirror due to spilling chemicals on it. This should be voided by only half filling the cups—more solution may be added later if ecessary.)

2. When the bottoms of the plungers are in contact with the bottoms of ne cups, the readings on both scales should be zero. If such is not the case, orrections should be made.

3. The depth of the layer of one of the solutions is usually set at a conenient point about midway on the scale. The height of the other cup is then djusted until the color intensities of the two fields match. Five to ten indeendent and successive adjustments should be made, and an average of the eadings used for calculations.

4. The two solutions must be perfectly clear and of similar color, or no omparison can be made.

5. Owing to the fact that many of the reactions performed in blood and rine analysis yield colored substances, the concentration of which depends n the temperature, time of heating or standing, concentration of reagents, H, and so forth, it is imperative that the standard and unknown be treated dentically. Also, since many of the colors formed require time to reach a naximum intensity and then fade, neither too short nor too long a period nust elapse before a comparison is made in the colorimeter.

6. Since the incident light is not monochromatic, the Lambert-Beer law applies only as a good approximation. It holds best when $c_1 = c_2$, for then both solutions are of the same concentration and all factors are equalized. For good results, the standard and unknown should have almost the same color intensity. Or, in other words, the readings on both scales should be close. If they are far apart, it is advisable to repeat the determination, using a standard which has a concentration nearer that of the unknown. This is especially true when the color-forming reaction is complex. For example, if one cup is set at 20, and if, upon obtaining a color match, the other cup is found to be lower than 10 or higher than 30, another standard should be used. Accurate results will not be obtained by diluting the deeper colored final solution, inasmuch as under these circumstances the two solutions will not have been treated identically. A shift in equilibrium may accompany the dilution.

PHOTOELECTRIC COLORIMETRY

The photoelectric colorimeter and the spectrophotometer have proved be valuable tools in quantitative analysis, particularly in biological chemist These instruments use photoelectric cells, either of the emissive or barr layer type, as a substitute for the eye. When light strikes such a cell, there a flow of electric current, which can be made to be directly proportional the light intensity. This current may be measured by a microammeter, galvanometer or, in the case of null point instruments, by a combination variable resistances and galvanometer. Photoelectric cells, though not qui so sensitive as the eye in detecting visible light, are far superior to it in the ability to match or make quantitative estimations of light intensities.

The advantages claim for the photoelectric method over visual metho of analysis are (1) elimination of the subjective factor inherent in visu comparative measurements, (2) greater accuracy, (3) extension of the rang

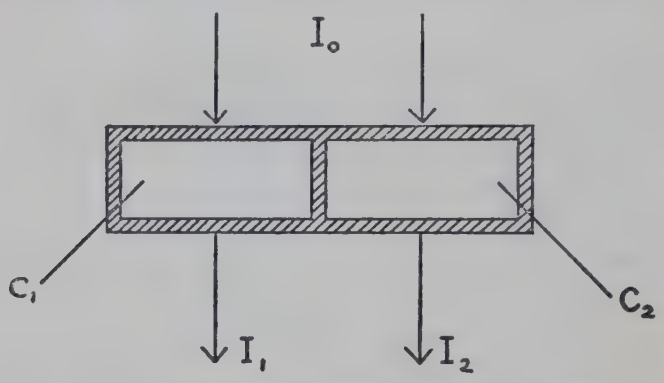

Fig. 12. Photoelectric measurement of intensity.

of colorimetric analysis to pale colors, and (4) determinations in the presence of interfering colors. With the exception of the first advantage, the others stem from the use of more nearly monochromatic light in this type of instru ment. To achieve this, the light from an incandescent tungsten source is first filtered through colored glass. On emerging, all but a comparatively narrow spectral region has been removed. In the case of spectrophotometers only a small portion of the tungsten spectrum is used as the incident light Thus the sensitivity of the instrument is enhanced and a stricter application of the Lambert-Beer law becomes possible.

In photoelectric colorimetry, as with visual colorimetry, the Lambert-Beer law is the basis for every exact method. If, in the relationship

$$\log_{10} \frac{I_0}{I} = kct$$

the concentration, c, is expressed as moles per liter, and the thickness of layer, t, in centimeters, then k becomes the *molar extinction coefficient* usually designated as ε. This coefficient is characteristic of the solution for incident light of any one wavelength.

In practice it is far simpler to achieve accuracy by comparing the intensity of the light transmitted by a standard solution (I_1) with that of the unknown

(I_2) (see Fig. 12). When the incident light intensity (I_0) is kept constant and the thickness (t) of layer of the liquids is the same, then

$$\log_{10} \frac{I_0}{I_1} = kc_1t \quad \text{and} \quad \log_{10} \frac{I_0}{I_2} = kc_2t$$

The relationship of I_0, I and c may be expressed in various forms

$$\log_{10} \frac{I_0}{I} = kc = E \text{ (extinction) or D (optical density)}$$

$$\frac{E}{t} = \varepsilon \text{ (extinction coefficient)}$$

$$\log_{10} \frac{I}{I_0} = -kc \text{ and}$$

$$\frac{I}{I_0} = T \text{ (transmittancy)}$$

$$\log_{10} T = -kc$$

When applied to calculations based on photocolorimetric measurements of unknown and a standard solution, the general equation is:

$$\frac{C_1}{C_2} = \frac{C_s}{C_u} = \frac{\log_{10} \frac{I_0}{I_s}}{\log_{10} \frac{I_0}{I_u}} = \frac{E_s}{E_u} = \frac{\log_{10} I_0 - \log_{10} I_s}{\log_{10} I_0 - \log_{10} I_u}$$

This may be expanded for convenient use to

$$C_u = \frac{R_u}{R_s} \times m_s \times \frac{100}{v} \times \frac{D_u}{D_s}$$

where C_u = milligrams of unknown per 100 ml. of blood
m_s = number of milligrams of the constituent in the standard solution
R_u = Optical density, or 2 — log of the transmittance reading of the unknown solution
R_s = Optical density, or 2 — log of the transmittance reading of the standard solution
v = volume of blood actually used in the color production
D_u = volume to which the unknown is diluted
D_s = volume to which the standard is diluted

EXAMPLE. For details of the methods used in this example, see Experiment 1 (Preparation of a Protein-Free Blood Filtrate, p. 79) and Experiment 8 (Determination of Creatinine, p. 95).

m_s = 0.030 mg. (5 × 0.0060 mg.)
v = 1.00 (10.00 ml. of filtrate = 1.00 ml. blood)
R_u = 1.30 (Optical density) or 50.1 per cent transmittance
R_s = 1.50 (Optical density) or 31.6 per cent transmittance
D_u = 15 (10.00 + 5.00)
D_s = 30 (5.00 + 15.00 + 10.00)

$$C_u = \frac{1.30}{1.50} \times 0.030 \times \frac{100}{1.00} \times \frac{15}{30} = 1.3 \text{ mg. per 100 ml.}$$

or

$$C_u = \frac{2 - \log_{10} 50.1}{2 - \log_{10} 31.6} \times 0.030 \times \frac{100}{1.00} \times \frac{15}{30}$$

$$= \frac{1.30}{1.50} \times 0.030 \times \frac{100}{1.00} \times \frac{15}{30} = 1.3 \text{ mg. per 100 ml.}$$

When it is expected that many determinations of the same constituer will be performed, instead of running a standard along with each unknow the photoelectric colorimeter may be calibrated. This is done by making up series of known solutions of the constituent—ranging from the lowest t the highest expected concentration. Each is treated in strict accordance wit the method. The transmittance of each is measured. From these data a calibra tion curve may be drawn by plotting the transmittancies directly against th concentrations. Other convenient choices of coordinates are shown in Figur 13, *D*, using semilogarithmic graph paper. Theoretically these latter curve are linear, and two points should fix the straight line. Actually, with mos instruments, especially when a large concentration range is covered, th curve deviates from a straight line, especially at the higher concentrations For this reason it is advisable to run many standards in order to fix th calibration curve.

Colorimeters using the barrier layer, self-generating type of photocell fal into two groups: the direct reading, single photocell type, and the null point double photocell type. A schematic diagram of the former type is shown in Figure 14. The light source is an incandescent tungsten filament which is kept at constant intensity by using a storage battery or voltage regulator as

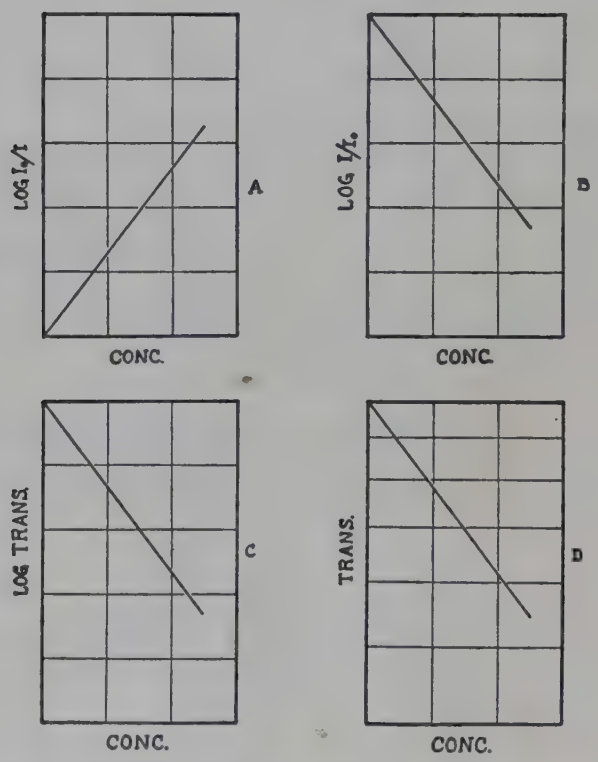

Fig. 13.

e current source. After passing through the chosen optical filter, the nergent light is somewhat more homogeneous in wavelength. Upon trav- sing the solution, which is placed in tubes or cuvettes of constant thick- ess, some light is absorbed; whatever is transmitted falls upon the photocell, enerating an electric current the magnitude of which is measured by a alvanometer or microammeter.

Fig. 14.

= battery; L = lamp; F = filter; A = tube containing solution; P = photoelectric cell; R_1R_2 = potentiometers; G = meter.

A schematic diagram of a double photocell colorimeter is shown in Figure 5. Various modifications of the bridge circuit have been used. In this case, ltered light from a single source reaches both photocells. If the cells are natched, there is no need to control the lamp voltage, since any light fluctua- ons affect both cells equally. The light, falling directly on one photocell, alled the "reference cell," causes a current to be generated. Light of the same nitial intensity passes through the colored solution before striking the second hotocell. This "working cell" also generates a current, but less than that of he "reference cell," since some light was absorbed while traversing the olution. By varying a resistance in the bridge circuit, the two currents may e balanced, the galvanometer acting as a null-point indicator. Since the ariable resistance is calibrated, the reading taken directly from it is a neasure of the transmittancy of the solution.

The procedure for the use of a photoelectric colorimeter is as follows: A suitable filter is chosen and placed in the light path. The light is switched on and the instrument given five to ten minutes to come to equilibrium. The eference solution—solvent or blank—is placed in a tube or cuvette. This is nserted in the colorimeter in the path of the filtered light. An adjustment is nade so that the meter reads full scale (100) for a direct reading instrument; or with a two-cell instrument the galvanometer is brought to its zero point n an appropriate manner. This is the I_0 value, often called 100 per cent ransmission. Without further changes the reference solution is immediately eplaced by the unknown, which is contained in an identical cuvette. The neter is read if it is a direct reading instrument. In the balanced circuit the ariable resistor is changed until the galvanometer once more reads zero leflection. The dial reading is taken. This is the I value. The process is re- peated for a second sample or for obtaining the value of the known standard olution.

The meter or dial graduations of various instruments differ. Some are inear; some logarithmic; some have both scales. If a linear scale is used,

the second reading is the transmittancy (T), sometimes called per c transmittancy. Then the relationship $\frac{C_u}{C_s} = \frac{\log_{10} T_0 - \log_{10} T_u}{\log_{10} T_0 - \log_{10} T_s}$ applies. If scale is logarithmic, the second reading is optical density (D). In this ca the concentrations are directly proportional to the readings if the Lambe Beer law holds and the relationship $\frac{C_u}{C_s} = \frac{D_u}{D_s}$ applies.

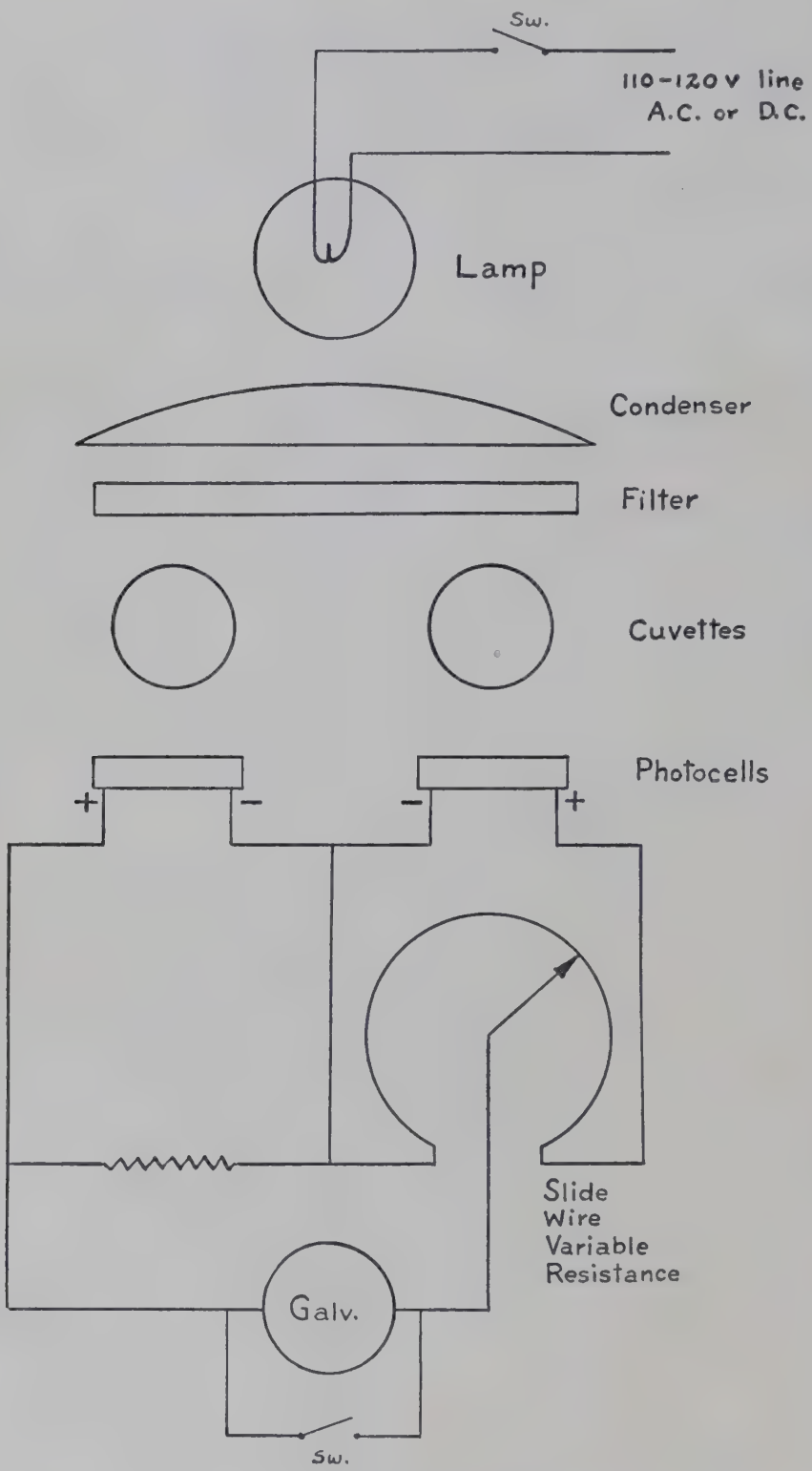

Fig. 15. Double photocell colorimeter.

For more detailed descriptions and directions in the use of any specific photoelectric colorimeter, the student is referred to the manufacturer's pamphlets.

SPECTROPHOTOMETERS

These instruments differ from filter photometers primarily in the source of the incident light. By means of a prism or a diffraction grating a spectrum

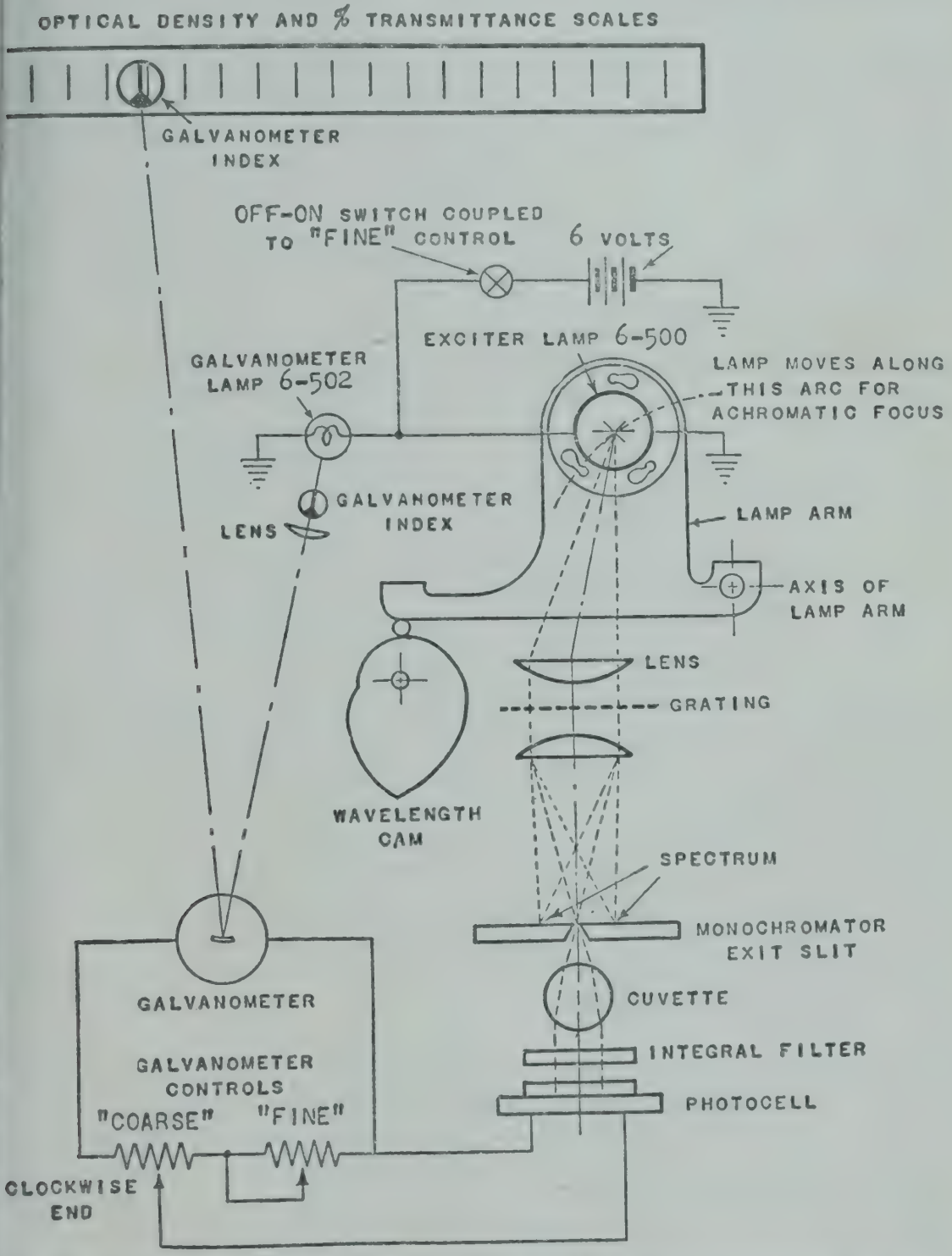

Fig. 16. Schematic diagram of Coleman junior clinical spectrophotometer optical and electrical systems.

is obtained, a small region of which is used for the incident light. The
persion, and so the "monochromaticity" of the light, depends on the
width in the optical system. By turning a calibrated dial, the chosen li
band is caused to pass through the solution, and so to the photocell
Fig. 16). The electrical system, the procedure for use, the calculations and
calibration are the same as for filter photometers.

FILTERS

The great advantage of photoelectric colorimeters is in their use of filte
The incident light thereby becomes a comparatively narrow spectral ba
Most of the filters used are made of glass. They are permanent and stab
The narrowness of the band of light transmitted by them depends on t
glass itself and its thickness. A combination of several different glasses
sometimes used to isolate a narrow spectral region. In general, at 50 per ce
transmission, the band width ranges from 25 to 75 millimicrons. Tod
interference filters are available that give an extremely narrow spectral ba
and yet have a very high transmission.

Greatest sensitivity is obtained when the transmittancy is measured wi
light whose wavelength coincides with the region of maximum absorpti
of the solution. Since most photoelectric colorimeters are provided with
limited number of filters, this condition may be approximated by choosi
one whose color is complementary to that of the solution.

Solution color	*Filter color*
Purple	Green
Orange to red	Blue to blue-green
Yellow	Blue
Blue	Red

Sometimes there is reason for not making this choice: for example, in th
determination of a colored constituent in the presence of an interfering colo
Here the filter of choice is a compromise between the one that yields light o
the wavelength absorbed least by the interference, and that absorbed most b
the constituent to be determined. Another exception is often made fo
practical purposes. When a method may not be varied and the resulting prod
uct is deeply colored, the use of the theoretically best filter may cause s
much light to be absorbed that photocell response is weak. In such a cas
better readings may be obtained if the color of the filter which is chose
approaches that of the solution.

Experiment 1. Absorption Spectra of Hemoglobin and Its Derivative
Determination of the exact wavelength for maximum absorption of light b
colored solutions is of importance in photocolorimetric determination
This information enables one to choose the proper filter in the case of photo
electric clorimeters like the Klett-Summerson or Leitz, or the correct wav
length in the case of spectrophotometers like the Beckman or Coleman.
also enables one to determine whether it is possible to distinguish amon
several colored constituents present together in the same solution. Dete

nination of the complete visible absorption spectra for hemoglobin and its derivatives serves to illustrate these problems.

OXYHEMOGLOBIN. Draw 5 ml. of blood using heparin as an anticoagulant. Centrifuge the cells, discard the plasma and wash twice by centrifugation with 10 ml. portions of 0.9 per cent saline. Dilute and lake the red cells with 500 times their volume of $M/15$ phosphate buffer, *p*H 6.6. Mix and centrifuge to clarify if necessary. Fill one cuvette with the oxyhemoglobin solution and another with the phosphate buffer which is to serve as the blank. Set the blank to 100 per cent transmission (zero density) and read the absorption of the oxyhemoglobin solution in terms of density. This is done for each wavelength setting from 400 to 700 mμ. Take readings at close intervals of wavelength near the maximum or minimum points of transmission. Plot the curve of density against wavelength.

REDUCED HEMOGLOBIN. Prepare Stokes' reagent as follows: to a mixture of 2 gm. ferrous sulfate and 3 gm. tartaric acid dissolved in 100 ml. of water, add ammonium hydroxide drop by drop until the precipitate which first forms completely dissolves. This is a reducing agent: ammonium ferro-tartrate. Add 2 drops of Stokes' reagent to 10 ml. of oxyhemoglobin solution. Add the same quantity to the blank. Repeat the determination of the absorption curve.

CARBOXYHEMOGLOBIN. Bubble gas containing CO, through a portion of the oxyhemoglobin solution for a few minutes. Use a well-ventilated hood. Repeat the determination.

METHEMOGLOBIN. Add 1 drop of freshly prepared 1 per cent solution of potassium ferricyanide to 10 ml. of the oxyhemoglobin solution. Treat the blank in the same manner. Repeat the determination.

CYANMETHEMOGLOBIN. Add 1 drop of a sodium cyanide solution to 10 ml. of the methemoglobin solution. Do the same for the blank used in the methemoglobin determination. Repeat the determination.

(*a*) Write the essential features of the chemical reactions.

(*b*) Of what practical value is the information derived from this experiment.

(*c*) From your curves, work out a method for the determination of methemoglobin in a specimen of blood. Check your results with the method reported by Evelyn and Malloy, J. Biol. Chem. *126*, 655 (1938).

Chapter 12 Determinations of Various Constituents in Blood

THE STANDARD, time-proven methods outlined in this chapter were selected with the view of acquainting the student with as large a variety of procedures as possible: volumetric, gasometric and colorimetric. Most of the colorimetric methods were originally designed for use with a visual colorimeter. They have not been modified. However, the wavelength at maximum light absorption (or the filter color) is noted in each case for the benefit of photocolorimeter users. In a few cases the coloration of the end products, under the conditions outlined, is so intense that the readings on some photoelectric colorimeters fall outside their range of maximum sensitivity. This occurs when the filter used is of low transmission and narrow spectral range. Then it is suggested that the instructor either modify the method by decreasing the concentration of the constituent, or by choosing a more appropriate filter. For example: In the Folin-Wu glucose determination, 2.00 ml. of tungstic acid blood filtrate are used. If it is found, when using the recommended red filter to measure the light intensity transmitted by the deep blue solution, that the photoelectric colorimeter readings are unsatisfactory, either 1.00 ml. of tungstic acid filtrate plus 1.00 ml. of water may be used, or a blue filter may be substituted for the red one.

Each of the colorimetric methods describes the procedure for the simultaneous reaction of an unknown and a standard. If it is desired to obtain a calibration curve when photoelectric colorimeters are used, a series of standards covering the expected range may be prepared and treated in the manner described for the standard. Explicit directions for preparing a calibration curve are given for the determinations of glucose, nonprotein nitrogen (p. 81), and sulfonamides (p. 101). These serve as examples of the procedure.

During the course of a five hour laboratory period each student can perform, in duplicate, the determination of one blood constituent. For this purpose he may be supplied with blood freshly drawn from an animal, e.g., a rabbit. The instructor can withdraw 25 ml. of blood by direct cardiac puncture, using heparin as an anticoagulant. By a slight modification of the amounts of whole blood needed for any determination, this quantity will suffice for a class of ten to twelve students. Besides the determination on blood, the student may also perform his experiment on an "unknown" solution to test his mastery of the technic.

The unknowns are aqueous solutions of the various substances being determined. Since they contain no protein, the usual procedure for the removal of proteins is omitted; instead the unknown is quantitatively diluted to the volume of the protein-free filtrate. This is necessary because the unknowns are made up in the same range of concentration as they are found

ı blood. For example: If a student is given a nonprotein nitrogen "unnown" solution containing 15 to 30 mg. per cent nonprotein nitrogen (*i.e.*, 3 to 86 mg. per cent urea), he transfers 5.00 ml. of this solution to a 50 ml. olumetric flask and dilutes it to mark. He then proceeds to use this diluted olution as the Folin-Wu tungstic acid protein-free filtrate.

The unknowns are most conveniently made up from a concentrated stock olution from which standards can be prepared as well.

Experiment 1. Preparation of a Protein-Free Blood Filtrate.* The lood is laked with dilute sulfuric acid. Upon adding sodium tungstate olution, tungstic acid is formed, which precipitates the proteins. Removal f the precipitate leaves a filtrate which contains only the nonprotein blood onstituents. The procedure is carried out quantitatively so that 1.00 ml. f filtrate is equivalent to 0.10 ml. of blood.

PROCEDURE. Mix the sample of blood in order to obtain a uniform suspension of cells.

Pipette 5.00 ml. of the well-mixed blood into a dry 125 ml. Erlenmeyer ask. (Allow the pipette to drain slowly.)

Add 40.0 ml. of $N/12$ sulfuric acid and mix well. (A burette is convenient or this addition.)

Add, drop by drop and with constant shaking of the flask, 5.00 ml. of 10 er cent sodium tungstate solution.†

Stopper and shake vigorously. (If conditions are right, no permanent oam should form, and the coagulum, which is at first pink, should change o brown. If the brown color does not develop, coagulation is incomplete. This may be due to an excess of anticoagulant or incorrect concentrations of he reagents.)

Allow the flask to stand for fifteen minutes, shaking at five minute ntervals.†

Have ready a dry, folded filter paper in a dry funnel, beneath which is a lry 125 ml. Erlenmeyer flask.

Pour a little of the mixture onto the double portion of the filter paper. When the whole paper has become wet, pour the remainder of the mixture nto the funnel.

Cover the funnel with a watch glass and collect the protein-free blood iltrate. (It should be perfectly clear and colorless. If the first small amount of the filtrate is cloudy, it should be refiltered.)

(*a*) What do we mean by "nonprotein nitrogen"?

(*b*) If protein precipitants other than alcohol are used, what substances other than proteins are carried down?

(*c*) What two protein precipitants are now most generally used?

(*d*) Name several other protein precipitants.

(*e*) What are some of the objections to (*d*)?

(*f*) What are some of the advantages of trichloracetic acid?

* Folin and Wu, *J. Biol. Chem.*, *38*, 81 (1919); Haden, *J. Biol. Chem.*, *56*, 469 (1923).

† If the quantity of available blood is small, ⅕ or ⅖ of the amounts of blood suggested ere may be used. Centrifuging the mixture instead of filtering it will result in a greater uantity of protein-free filtrate.

(*g*) Why is the filtrate from the tungstic acid nearly neutral?

(*h*) Why must the solution of the 10 per cent sodium tungstate be neith too acid nor too alkaline?

(*i*) Why is it necessary to shake the flask constantly on adding the ac and tungstate?

(*j*) What is the principal source of error in this procedure?

(*k*) In what determinations is it necessary to use a protein precipita other than tungstic acid?

REAGENTS:

10 PER CENT SODIUM TUNGSTATE SOLUTION. Dissolve 100 gm. of C.P sodium tungstate in 900 ml. of water. This solution should be neutral t phenolphthalein; if necessary, add sodium hydroxide or sulfuric acid t make it so.

N/12 SULFURIC ACID. Add 2 ml. of concentrated sulfuric acid (18 *N* to 862 ml. of water in a liter measuring cylinder. Mix well and transfer to clean, dry bottle. Check the normality by titration with 0.1 *N* sodiu hydroxide.

Experiment 2. Determination of Nonprotein Nitrogen (Folin-Wu). Protein-free Folin-Wu blood filtrate is heated with sulfuric acid until th water has been boiled off. Further digestion decomposes the nitrogenou substances, such as urea, uric acid, amino acids, and so forth, converting th nitrogen quantitatively into ammonia which is fixed by the acid in the form of ammonium sulfate. Hydrogen peroxide is used to complete the oxidatio of organic matter. After dilution, Nessler's solution is added. It forms wit ammonia an orange compound which remains in solution for a short time The color developed is a measure of the ammonia and therefore of the nitrogen present.

PROCEDURE. Make a protein-free Folin-Wu blood filtrate (see p. 79).

Transfer 5.00 ml. of the tungstic acid filtrate to a 200 by 25 mm. Pyrex test tube graduated at 35 and 50 ml.

Add 1 ml. of 50 per cent sulfuric acid and a single clean glass bead.

Clamp the tube on a ring stand at an angle of about 60 degrees and heat gently to evaporate the water. Then boil vigorously in the full flame of a micro-burner until characteristic dense white fumes fill the tube, keeping bumping at a minimum by cautious manipulation of the flame. (Perform the digestion in the hood!)

Remove the burner and allow the tube to cool for about five minutes.

Adjust the clamp so that the tube is in a vertical position. Add 2 or at most 3 drops of 30 per cent hydrogen peroxide (superoxol) so that it falls directly into the liquid in the tube. **(Caution: This procedure may cause spattering.)**

Incline the tube to an angle of 60 degrees and heat again until character-istic dense white fumes fill the tube.

If the brown color of the solution fails to disappear, repeat the addition of a drop or two of the peroxide and subsequent boiling. (Further heating

* Folin and Wu, *J. Biol. Chem.*, *38*, 81 (1919); Koch and McMeekin, *J. Am. Chem. Soc.*, *66*, 2066 (1924).

ten produces a yellow precipitate which does not disappear on prolonged gestion. Since this precipitate will dissolve on "nesslerizing," it does not terfere with the determination.)

Finally, boil for one minute after the solution becomes colorless, and then ol to room temperature or below.

Into a second digestion tube, graduated at 35 and 50 ml., transfer 3.00 l. of standard ammonium sulfate solution. Add to it 1 ml. of the 50 per nt sulfuric acid used previously.

****Dilute all the solutions to the 35 ml. graduation with ammonia-free ater.

Add to each 3 drops of gum ghatti solution.

Add sufficient Nessler's solution to each tube so as to bring the level of e liquid to the 50 ml. mark.

Immediately stopper each tube and mix the contents by inverting several mes.

Read in the colorimeter from five to twenty minutes after the addition of essler's reagent.

Calculate the number of milligrams of nonprotein nitrogen per 100 ml. of ood. When using the visual colorimeter, refer to the equations on page 67. a photoelectric colorimeter is used, the appropriate relationships may be und on page 71, or a calibration curve may be used.

CALIBRATION CURVE. Place six digestion tubes, graduated at 35 and 50 l., in a rack. Pipette into them the quantities of standard ammonium sulfate olution listed in the table below.

Tube Number	*Milliliter of Dilute Standard Ammonium Sulfate Solution*	*Milligram Per Cent Nitrogen*
1	0.00	0
2	1.50	20
3	3.00	40
4	6.00	80
5	9.00	120
6	12.00	160

Continue exactly as above for the unknown from the asterisks on.

Read in a photoelectric colorimeter, using the appropriate filter. One with naximal transmission in the blue at 420 mμ will give good results.

Plot the data on linear or semilog graph paper.

REAGENTS:

50 PER CENT SULFURIC ACID. Cautiously mix 1 volume of concentrated, nitrogen-free sulfuric acid with 1 volume of distilled water.

30 PER CENT HYDROGEN PEROXIDE (Perhydrol or Superoxol).

STOCK NESSLER'S SOLUTION. Dissolve 25 gm. of iodine in 20 ml. of water containing 30 gm. of potassium iodide. After the solution is complete dd 30 gm. of redistilled metallic mercury, and shake the mixture well. (If becomes too hot, cool in running tap water.) Continue shaking until the upernatant liquid has lost all the yellow color due to the iodine. Filter the olution through glass wool. Wash the mercury, sediment and flask with nall quantities of distilled water and add the washings to the solution.

(The solution should be clear.) To about 1 ml. of a 1 per cent soluble star solution in a test tube, add a few drops of the solution. If a positive test iodine is not obtained, add, drop by drop, an iodine solution of the sa composition as used before, until a faint excess of iodine can be detect Dilute to 200 ml. and mix well. To 975 ml. of an accurately prepared 10 p cent sodium hydroxide solution add the entire solution of potassium mercu iodide prepared above. Mix thoroughly and allow to stand.

Stock Ammonium Sulfate Solution. Weigh 0.9433 gm. of pu anhydrous ammonium sulfate and quantitatively transfer it to a 1 lit volumetric flask, using *N*/5 sulfuric acid for washing purposes. Dilute mark with the *N*/5 sulfuric acid. Mix well and transfer it to a clean, d bottle. This solution contains 0.20 mg. of nitrogen per milliliter.

Dilute Standard Ammonium Sulfate Solution. Pipette 20.00 ml. the above stock solution into a 1 liter volumetric flask. Add 180 ml. of *N*/ sulfuric acid. Dilute to mark with ammonia-free water. Mix thoroughly a transfer to a clean, dry bottle. This solution is permanent and contains 0.0 mg. of nitrogen per milliliter.

Gum Ghatti Solution. Fill a liter cylinder with distilled water. Pla 20 gm. of soluble gum ghatti in a gauze bag and suspend it just below t surface of the water. Allow to stand overnight, but not longer than twen hours. Remove the gauze bag, which may contain undissolved materia Strain the solution through several thicknesses of gauze. Dissolve 1 gm. benzoic acid in 10 ml. of alcohol and then add it to the gum ghatti solutio Mix thoroughly and transfer to an appropriate container. The solution kee for months if stored in a refrigerator.

N/5 Sulfuric Acid. Dilute 5.6 ml. of concentrated sulfuric acid (3 *N*) to 1 liter.

Experiment 3. The Determination of Nonprotein Nitrogen (Micro Kjeldahl).* The nitrogenous compounds of a protein-free blood filtrat are decomposed and converted to ammonium sulfate by digestion wit sulfuric acid, copper sulfate catalyzing the reaction. The ammonia is liberate by making the solution alkaline, and then it is steam distilled into a saturate solution of boric acid. The resulting distillate is titrated with standard acid This is possible because boric acid is so weak that it does not appreciabl dissociate in the *p*H range of methyl red (4.2 to 6.4). Ammonia, which i extremely soluble in water, is held in solution more securely in the presenc of boric acid. Ammonium borate, on the other hand, is almost completel hydrolyzed in aqueous solution. Therefore the ammonia which was distille from the alkaline digestion mixture may be quantitatively titrated with min eral acid to *p*H 5.6 without interference by the boric acid.

Procedure. Make a protein-free, Folin-Wu blood filtrate (see p. 79)

Pipette 20.0 ml. of the tungstic acid blood filtrate into a micro-Kjeldah digestion flask.

Add 2 ml. of concentrated sulfuric acid, a pinch of copper sulfate-potas sium sulfate mixture, and a clean glass bead.

* Pregl, Quantitative Microanalysis (1930), p. 109; Sobel, Yuska and Cohen, *J. Bio Chem.*, *118*, 443 (1937).

Evaporate the water over a small flame, rotating the flask. Continue heating until heavy white fumes of the oxides of sulfur are evolved. Remove the flame and add 2 or 3 drops of superoxol. Continue the digestion on the digestion rack ten to fifteen minutes longer.

Some time before, or while the digestion is being carried out, half-fill flask *A* (see Fig. 17) with water to which is added a few drops of concentrated sulfuric acid and 2 ml. of 0.2 per cent methyl red indicator. Heat the water in flask *A* to boiling and steam out the apparatus for ten to fifteen minutes. Wash down *G* (the end of the condenser) with distilled water. Run water through the condenser. The apparatus is now ready for use.

When digestion is complete, cool the digestion flask, rinse down the sides

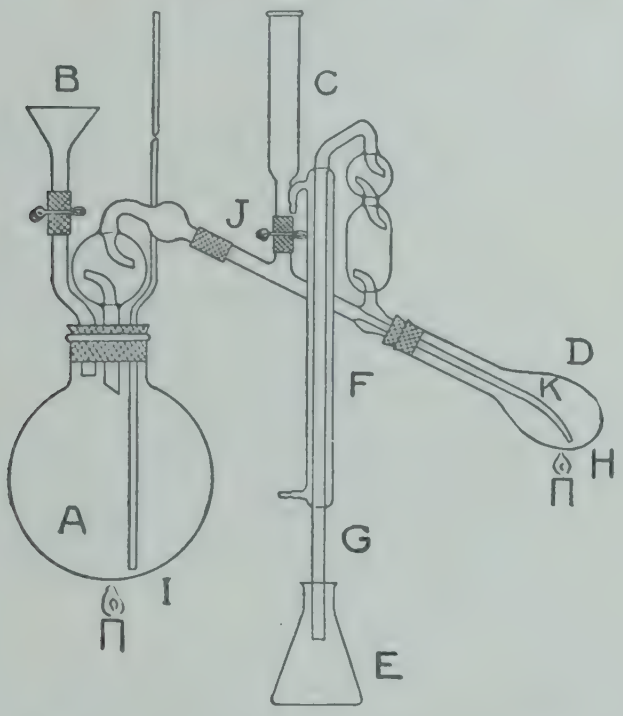

Fig. 17. Modified micro-Kjeldahl apparatus.

of the flask with 10 ml. of distilled water. **(Caution!)** Cool under running tap water.

Measure into a 125 ml. Erlenmeyer flask, 5 ml. of saturated boric acid solution and 5 ml. of water. Add one drop of the mixed indicator.

Before attaching the digestion flask, containing the digested sample, run a blank by using a Kjeldahl flask containing 2 ml. of concentrated sulfuric acid, a pinch of copper sulfate-potassium sulfate and a clean glass bead.

Clamp the digestion flask containing the digested sample (or blank) onto the apparatus at position *D*.

Place the Erlenmeyer flask containing the boric acid solution under the condenser at position *E*, so that the tip of the latter dips well into the solution. It is best to tilt the flask in order to increase the depth of acid through which the ammonia will pass.

Open the pinch clamp at *J* and allow 15 ml. of the concentrated alkali to flow slowly into the flask, forming a layer beneath, but not mixing with the acid solution.

Place the burner, *I*, under the steam generating flask and heat to boili
Place a small flame, *H*, under the digestion flask to prevent excessive c
densation.

Steam distil the ammonia from the digestion flask for five minutes,
until about 40 ml. has been collected.

Lower the Erlenmeyer flask so that the tip of the condenser is abou
inch above the level of the liquid in the flask. Continue steam distilling
one minute.

Wash the tip of the condenser, collecting the washings in the flask.

Remove the flames from under both flasks.

Titrate the ammonia absorbed by the boric acid solution with standa
0.01000 *N* hydrochloric acid, using a 10 ml. burette. Titrate to the sa
color as obtained in the titration of the blank.

Remove the digestion flask. The apparatus is now ready for the ne
determination.

CALCULATIONS. $A \times 0.14 \times 50 =$ mg. N. P. N. per 100 ml. of bloo
$A =$ ml. of 0.01000 *N* hydrochloric acid used to titrate the ammonia.

REAGENTS:

MIXED INDICATOR. Mix 1 volume of a 0.2 per cent alcoholic solution
methyl red with 1 volume of a 0.1 per cent alcoholic solution of methyle
blue. The methylene blue is present merely to make the color change mo
easily discernible. At *p*H 5.2 the mixed indicator is red; at *p*H 5.4 colorles
and at *p*H 5.6 it is green.

HYDROCHLORIC ACID STANDARD. 0.01000 *N*. Diluted from accurate
standardized 0.1 *N* hydrochloric acid.

CONCENTRATED SULFURIC ACID. This must be ammonia-free.

COPPER SULFATE-POTASSIUM SULFATE. One part of potassium sulfa
to three parts of copper sulfate.

ALKALI. 30 per cent sodium hydroxide.

BORIC ACID SOLUTION. Saturated water solution. It should react acid t
methyl red, and 1 drop of 0.01 *N* sodium hydroxide should discharge th
red color when added to 5 ml. of the solution.

(*a*) Why is a determination of nonprotein nitrogen on plasma mor
important than on whole blood?

(*b*) What constitutes nonprotein nitrogen?

(*c*) What have been some improvements over Kjeldahl's original pro
cedure?

(*d*) Is the Kjeldahl method applicable to all nitrogenous compounds

(*e*) Summarize the three methods usually adopted for estimating th
ammonia nitrogen in the digest.

(*f*) What other indicators could be used instead of methyl red?

(*g*) Why does the tip of the condenser have to dip into the boric aci
during the first part of the distillation?

(*h*) Why is the flask lowered from the tip of the condenser at the end o
the distillation?

(*i*) Why are indicator and sulfuric acid added to the steam generator

(*j*) Why is the digestion flask cooled before water is added to the diges
tion mixture?

(*k*) Why must a specially designed digestion flask be used in the Kjeldahl determination?

(*l*) Derive the formula for the calculation of the results.

Experiment 4. The Determination of Glucose (Folin-Wu).* Protein-free, Folin-Wu blood filtrate is heated with an alkaline copper tartrate

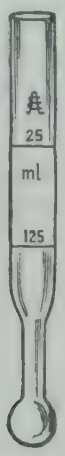

Fig. 18. Folin-Wu blood sugar tube.

solution; the glucose reduces the cupric ion to insoluble cuprous compounds. Upon the addition of phosphomolybdic acid reagent, the cuprous compounds dissolve and at the same time reduce the faintly colored phosphomolybdate ion to a lower valence state, yielding densely blue, soluble oxides of molybdenum. The intensity of the color of the "molybdenum blue" is a measure of the copper reduced to the cuprous condition and therefore of the amount of the glucose present.

$$C_6H_{12}O_6 + Cu^{++} \longrightarrow Cu^{+}$$
$$Cu^{+} + Na_3PO_4.12Mo^{6+}O_3 \longrightarrow Cu^{++} + 12Mo^{<6} \text{ (blue)}$$

PROCEDURE. Make a protein-free, Folin-Wu blood filtrate (see p. 79).

Pipette 2.00 ml. of the tungstic acid blood filtrate to a Folin-Wu blood sugar tube (Fig. 18).

Transfer 2.00 ml. of the dilute glucose standard No. 1 to a second blood sugar tube.

****Add 2.00 ml. of alkaline copper tartrate reagent to each tube. (The surface of the mixture should now have reached the middle of the constricted part of the tube.)

Mix the contents of the tubes by shaking laterally.

Place the tubes in an actively boiling water bath and heat for exactly six minutes.

Remove the tubes and cool them at the same time by placing them in a beaker of cold water for three minutes.

Add to each tube 2.0 ml. of the phosphomolybdic acid reagent.

* Folin and Wu, *J. Biol. Chem.*, *41*, 367 (1920); Benedict, *J. Biol. Chem.*, *92*, 141 (1931); Schirmer, Andrieth, Gross, McClellan and Sippi, *J. Am. Chem. Soc.*, *64*, 2543 (1942), describe the composition of molybdenum blue.

Mix the solutions by gentle shaking. (The cuprous oxide should dissol immediately.)

Dilute the solutions with water to the 25 ml. mark.

Stopper each tube and mix the contents thoroughly by inverting seve times.

Compare immediately in a colorimeter.

Calculate the number of milligrams of glucose per 100 ml. of blood. Wh using the visual colorimeter, refer to the equations on page 67. If a phot electric colorimeter is used, the appropriate relationships may be found page 71, or a calibration curve may be used.

CALIBRATION CURVE. Place seven labelled Folin-Wu sugar tubes in rack. Pipette into them the quantities of standard glucose solutions a distilled water listed in the table below.

Tube Number	*Ml. Standard No. 1*	*Ml. Standard No. 2*	*Ml. Water*	*Mg. per ce*
1.....	0.0	...	2.0	0
2.....	0.5	...	1.5	50
3.....	1.0	...	1.0	100
4.....	1.5	...	0.5	150
5.....	2.0	...	..	200
6.....	...	1.5	0.5	300
7.....	...	2.0	..	400

Continue as above for the unknown from the asterisks on.

Read in the photoelectric colorimeter within ten minutes using the appro priate filter. One with maximal transmission in the orange at 580 mμ wi give good results.

Prepare a calibration curve on linear or semilog graph paper by plottin the data.

REAGENTS: ALKALINE COPPER TARTRATE SOLUTION. Dissolve 40 gm. o pure anhydrous sodium carbonate in about 400 ml. of water and transfer to 1 liter volumetric flask. Add 7.5 gm. of tartaric acid and, when the latter ha dissolved, add 4.50 gm. of crystalline copper sulfate ($CuSO_4.5H_2O$). Mi and dilute to mark. Transfer to a clean, dry, amber bottle.

PHOSPHOMOLYBDIC ACID REAGENT. To 35 gm. of molybdic acid and gm. of sodium tungstate, add 200 ml. of 10 per cent sodium hydroxide an 200 ml. of water. Boil in a beaker for thirty minutes, keeping the volum approximately constant by the addition of water from time to time. Cool t room temperature and transfer the solution to a 500 ml. volumetric flask. Ad 125 ml. of concentrated phosphoric acid (syrupy, 85 per cent). Mix, dilut to mark, and transfer to an amber bottle.

SATURATED BENZOIC ACID. Make an aqueous saturated benzoic aci solution.

STOCK 1 PER CENT GLUCOSE SOLUTION. Carefully weigh 1.000 gm of pure glucose. Transfer to a 100 ml. volumetric flask, using a saturate benzoic acid solution for washing purposes. Then dilute to mark with th same solvent. Transfer the solution to a dry, amber bottle. (If crystals o benzoic acid settle out on cooling, ignore them.)

Dilute Standard Glucose Solution No. 1. Pipette 2.00 ml. of the ock 1 per cent glucose solution into a 100 ml. volumetric flask. Dilute to ıark with saturated benzoic acid solution. Stopper the flask and mix its con-nts thoroughly. This solution, which should be made up fresh from time) time, contains 0.20 mg. of glucose per milliliter.

Dilute Standard Glucose Solution No. 2. Pipette 4.00 ml. of the ock 1 per cent glucose solution into a 100 ml. volumetric flask. Dilute to ıark with saturated benzoic acid solution. Stopper the flask and mix its ontents thoroughly. This solution, which should be made up fresh from me to time, contains 0.40 mg. of glucose per milliliter.

Experiment 5. The Determination of Glucose (Nelson).* The blood roteins are precipitated by means of a mixture of zinc sulfate and barium ydroxide, which produces zinc hydroxide. The two reagents are so balanced ıat no excess of either remains in the solution. The barium sulfate and zinc ydroxide are both insoluble, thus allowing a complete removal of proteins ith no interference by excess foreign reagents or change in *p*H. The lucose is determined by heating with alkaline copper reagent, and the uprous oxide formed is treated with an arsenomolybdate reagent, which is educed to a colored compound whose intensity is proportional to the cuprous on and thus to the glucose present.

Procedure. To 1.00 ml. of whole blood add 15.00 ml. of water and ix. Add 2.00 ml. of barium hydroxide solution and mix. When the mix-ure has turned brown, add 2.00 ml. of zinc sulfate solution. Mix.

After a few minutes, filter (or centrifuge and then filter to obtain a larger olume of filtrate).

Place 1.00 ml. of the filtrate into a Folin-Wu tube graduated at 25 ml. see p. 85). Add 1.00 ml. of a fresh mixture of reagents *A* and *B*.

In a similar tube graduated at 25 ml. place 1.00 ml. of the glucose stand-rd solution containing 0.05 mg. of glucose per milliliter and add 1.00 ml. f the mixed solution of reagents *A* and *B*.

Place the tubes in an actively boiling water bath for twenty minutes.

Cool in a beaker of cold water and add 1.0 ml. of the arsenomolybdate eagent to each tube. Dilute to the 25 ml. mark with water, stopper, mix, nd read the color at any convenient time. The color is stable.

For the photoelectric colorimeter, a filter which absorbs at 500 or 520 ıμ is used, and a blank is run as for the standard, with the omission of the tandard glucose solution.

Reagents:

Copper Reagent A. To a 1 liter volumetric flask add 25 gm. of nhydrous sodium carbonate, 25 gm. of Rochelle salts, 20 gm. of sodium icarbonate and 200 gm. of anhydrous sodium sulfate, and dissolve with ater. Dilute to the mark. Filter if necessary and store at a temperature not ower than 20°C.

Copper Reagent B. Transfer 15 gm. of $CuSO_4.5H_2O$ to a 100 ml.

* Nelson, *Biol. Chem. 153*, 375 (1944).

volumetric flask, dissolve with water and add 2 drops of concentra sulfuric acid. Dilute to mark. To prepare a mixture of the two reagents, m 25 parts of *A* with 1 part of *B*.

ARSENOMOLYBDATE REAGENT. Dissolve 25 gm. of ammonium mol date in 400 ml. of water. Add 21 ml. of concentrated sulfuric acid and m Add 3 gm. of $Na_2HAsO_4.7H_2O$ dissolved in 25 ml. of water. Mix and pl in an incubator at 37°C. for twenty-four to forty-eight hours. Store in amber glass-stoppered bottle.

ZINC SULFATE. Dissolve 50 gm. of $ZnSO_4.6H_2O$ in 1 liter of water.

BARIUM HYDROXIDE, APPROXIMATELY 0.3 *M*. Dissolve 51.5 gm. of anhydrous base (or an equivalent amount of the hydrate) in water to ma 1 liter of solution. Store in a bottle protected with a soda lime tube. Adj with water so that 5 ml. of the zinc sulfate solution requires 4.7 to 4.8 m of the barium hydroxide solution to produce a definite pink color in t presence of phenolphthalein after the addition of 15 ml. of water.

Experiment 6. The Determination of Glucose (Shaffer-Hartmann*) Hemolyzed blood is deproteinized with zinc hydroxide, yielding a filtrate which the glucose is the sole substance reduced under the prescribed co ditions. The filtrate is heated with an excess of alkaline tartrate reage thereby reducing part of the cupric ions to cuprous compounds. Upon aci ifying, an excess of iodine is liberated, since the reagent contains pota sium iodide and potassium iodate. The cuprous compounds are thus reco verted to the cupric state. The excess iodine is titrated with standardize thiosulfate solution. From these data the amount of glucose initially prese may be determined by reference to a table (see p. 91). Since the amou of thiosulfate used is not strictly proportional to the amount of glucos originally present, the table was drawn up so that elaborate calculatio would not be necessary.

$$Cu^{++} + C_6H_{12}O_6 \longrightarrow Cu^{+} + \text{unknown oxidation products of } C_6H_{12}O_6$$
$$KIO_3 + 5KI + 3H_2SO_4 \longrightarrow 3I_2 + 3K_2SO_4 + 3H_2O$$
$$Cu_2O + I_2 + H_2SO_4 \longrightarrow CuSO_4 + CuI_2 + H_2O$$

Cupric iodide (CuI_2) normally tends to decompose into cuprous iodide an iodine.

$$2CuI_2 \longrightarrow 2CuI + I_2$$

In the presence of excess tartrate, however, the equilibrium is held so tha the cupric iodide which is formed does not decompose, and the reactio proceeds quantitatively.

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

PROCEDURE. Pipette 5.00 ml. of well-mixed blood into a dry 125 m Erlenmeyer flask. (Allow the pipette to drain slowly.)

Add 35.00 ml. of water to the blood in the flask and mix well to assu hemolysis.

* Shaffer and Hartmann, *J. Biol. Chem.*, *45*, 365 (1921); Somogyi, *J. Biol. Chem.*, 7 599 (1927).

Add 5.00 ml. of 10 per cent zinc sulfate solution and mix again.

Add slowly, drop by drop, and with constant shaking of the flask, 5.00 ml. of $N/2$ sodium hydroxide solution.

Stopper and shake vigorously. (If conditions are right, no permanent foam or bubbles should form.)

Allow the flask to stand for five minutes.

Place a dry, folded filter paper in a dry funnel beneath which is a dry 125 ml. Erlenmeyer flask.

Pour a little of the mixture onto the double portion of the filter paper. When the whole paper has become wet, pour the remainder of the mixture into the funnel.

Cover the funnel with a watch glass and collect the protein-free filtrate. It should be perfectly clear and colorless. If the first small amount of the filtrate is cloudy, pour it back into the funnel for refiltration.)

Pipette into a large test tube (25 by 250 mm.) 5.00 ml. of the alkaline copper reagent.

Add 5.00 ml. of the protein-free filtrate.

Mix by gentle shaking and place a 50 ml. beaker over the mouth of the tube.

Place the tube in an actively boiling water bath and allow it to remain in the bath for exactly fifteen minutes.

Remove the tube and, without shaking it, place it in a beaker of cold water for about three minutes.

When its temperature has fallen to 35°C., remove the tube from the cold water and, without shaking, add 5 ml. of N sulfuric acid.

Shake well, making sure that all the precipitated cuprous oxide dissolves.

After about two minutes, titrate the contents of the tube with approximately 0.005 N thiosulfate solution until almost all the yellow color disappears.

Add 2 ml. of the 1 per cent starch solution and continue the titration until the blue color due to the starch-iodine complex disappears. (Remember that the solution has a faint blue color due to cupric ions.)

A blank titration (which may be run at the same time as the unknown) is made by substituting 5 ml. of water for the 5 ml. of protein-free filtrate and repeating the procedure just outlined.

Standardize the thiosulfate solution by measuring out exactly 5.00 ml. of the standard 0.01000 N potassium iodate solution into a 125 ml. Erlenmeyer flask, adding 2 ml. of N sulfuric acid and titrating with the approximately .005 N thiosulfate solution until the yellow color due to the liberated iodine almost disappears. Then add 2 ml. of the 1 per cent starch indicator solution and continue the titration until the blue color due to the starch-iodine complex disappears.

(This procedure may be used to determine glucose in urine. The urine is diluted so that 5 ml. contained about 0.1 to 0.2 mg. of glucose. Five milliliters of the diluted urine are substituted for the protein-free filtrate. The dilution, of course, must be noted.)

CALCULATIONS:

$$\frac{(A - B)10}{C} = \text{ml. of 0.00500 } N \text{ thiosulfate used.}$$

A = ml. of approximately 0.005 N thiosulfate for blank
B = ml. of approximately 0.005 N thiosulfate for unknown sample
C = ml. of approximately 0.005 N thiosulfate for standardization wi
5.00 ml. of 0.01000 N iodate

The value obtained is referred to Table 3, where the milligrams of glucos per 100 ml. of blood are read off directly.

REAGENTS:

ALKALINE COPPER REAGENT. Weigh 25 gm. of Rochelle salt, 25 gm of anhydrous sodium carbonate and 20 gm. of sodium bicarbonate. Transfe these salts to a 1 liter volumetric flask and dissolve them completely by ad ing approximately 600 ml. of warm water, and shaking. Weigh out 7.5 gm of crystalline copper sulfate. Dissolve the salt by adding about 100 ml. water, warming if necessary. Transfer this solution to the volumetric flas Mix. Weigh 5 gm. of potassium iodide, transfer to the volumetric flask an dissolve the substance. Accurately weigh 0.785 gm. of potassium iodat into a small beaker and dissolve in about 50 ml. of water. Quantitativel transfer this solution to the volumetric flask. Shake well. Allow to come t room temperature and add water to mark. Transfer to a clean, dry, ambe bottle. (Instead of potassium iodate, one may substitute 22.00 ml. of a alkaline standard normal solution of potassium biiodate. The latter solutio contains 32.498 gm. of potassium biiodate and 83.3 ml. of N sodiu hydroxide solution per liter of solution.)

STOCK SOLUTION OF APPROXIMATELY 0.1 N THIOSULFATE. Weigh 2 gm. of sodium thiosulfate ($Na_2S_2O_3.5H_2O$) and transfer to a 1 liter volu metric flask. Dissolve the salt in about 500 ml. of water. Add 5 ml. of sodium hydroxide. This increases the stability of the solution. Make up t mark. Transfer to a clean, amber bottle.

STOCK SOLUTION OF APPROXIMATELY 0.005 N THIOSULFATE. Transfe 50 ml. of the stock 0.1 N thiosulfate solution to a 1 liter volumetric flask Add 2 drops of 10 per cent sodium hydroxide and dilute to mark. Transfe to an amber bottle. This solution must be standardized.

STANDARD 0.01000 N POTASSIUM IODATE SOLUTION (0.01000 N IODINE) Accurately weigh 0.3567 gm. of potassium iodate. Transfer it quantita tively to a 1 liter volumetric flask. Weigh out 1.4 gm. of potassium iodid and transfer it to the flask. Dissolve the salts in 500 ml. of water. Make up to mark. Transfer the solution to a dry, amber bottle.

1N SULFURIC ACID. Dilute 27.8 ml. of concentrated sulfuric acid (36 N) to 1 liter.

1 PER CENT STARCH SOLUTION. Weigh, into a 100 ml. beaker, 5 gm. of soluble starch. With a minimum amount of water, make a thin, uniform paste of the starch by stirring the mixture thoroughly. Bring 500 ml. of water to the boiling point and add the starch paste slowly, with continuous stirring. Boil the solution for a minute. Cool. Transfer to a clean bottle. Preserve by adding a few crystals of mercuric iodide and a drop of toluene.

10 Per Cent Zinc Sulfate Solution. Dissolve 100 gm. of hydrated nc sulfate ($ZnSO_4.7H_2O$) in 1 liter of water. Transfer to a clean bottle.

N/2 Sodium Hydroxide. Dilute 30 ml. of saturated sodium hydroxide lution to 1 liter. Standardize.

(*a*) Why is a strictly accurate method for glucose not possible when ngstic acid is used as a precipitant for blood?

(*b*) How can the situation in (*a*) be remedied?

Table 3

MOUNTS OF GLUCOSE CORRESPONDING TO TITRATION VALUES WHEN 5 ML. OF 1: 10 BLOOD FILTRATE AND 5 ML. OF ALKALINE COPPER REAGENT ARE HEATED IN A WATER BATH FOR FIFTEEN MINUTES

Ml. of 0.00500 *N* Thiosulfate (A—B)10/C	*Tenths of 1 Ml. of 0.00500* N *Thiosulfate*									
	0	1	2	3	4	5	6	7	8	9
	Milligrams of Glucose in 100 Ml. of Blood									
0.....	....	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0	27.0
1.....	29.5	32.0	34.5	37.0	39.0	41.5	44.0	46.0	48.5	50.5
2.....	53.0	55.0	57.5	59.5	62.0	64.0	66.5	69.0	71.0	73.0
3.....	75.5	78.0	80.0	82.0	84.5	87.0	89.0	91.5	93.5	96.0
4.....	98.0	100.0	102.5	105.0	107.0	109.0	111.5	113.5	115.5	118.0
5.....	120.0	122.5	125.0	127.0	129.0	131.5	134.0	136.0	138.0	140.5
6.....	143.0	145.0	147.0	149.0	151.0	154.0	156.0	158.0	160.0	163.0
7.....	165.0	167.0	169.0	171.0	174.0	176.0	178.0	180.0	182.0	185.0
8.....	187.0	189.0	191.0	194.0	196.0	198.0	200.0	202.0	204.0	207.0
9.....	209.0	211.0	213.0	215.0	218.0	220.0	222.0	224.0	226.0	229.0
10.....	231.0	233.0	235.0	237.0	240.0	242.0	244.0	246.0	248.0	251.0
11.....	253.0	255.0	257.0	260.0	263.0	265.0	267.0	269.0	271.0	274.0
12.....	276.0	278.0	280.0	283.0	285.0	287.0	290.0	292.0	294.0	296.0
13.....	298.0	300.0	303.0	305.0	307.0	309.0	312.0	314.0	316.0	318.0
14.....	320.0	322.0	325.0	327.0	329.0	331.0	334.0	336.0	338.0	340.0
15.....	342.0	344.0	347.0	349.0	351.0	353.0	355.0	358.0	360.0	363.0
16.....	364.0	366.0	369.0	371.0	373.0	375.0	377.0	380.0	382.0	384.0
17.....	386.0	388.0	390.0	392.0	394.0	396.0	398.0	400.0		

(*c*) Upon what properties of glucose do methods for its determination lepend?

(*d*) What are some of the oxidizing agents used?

(*e*) What metals other than copper have been used?

(*f*) What grouping in the sugar molecule causes the reduction of cupric salts?

(*g*) What are some of the interfering substances?

(*h*) Why is starch indicator used only after most of the iodine has eacted?

(*i*) Why cannot the sodium thiosulfate solution be used as the primary tandard?

(*j*) Why is sulfuric acid added to the copper oxide?
(*k*) Why are Rochelle salts added to the copper reagent?
(*l*) Why is sodium carbonate added to the copper reagent?
(*m*) Why are the Folin-Wu sugar tubes constricted?
(*n*) Why is the glucose standard made up in saturated benzoic acid?
(*o*) Derive the formulae used for calculating the results.

Experiment 7. The Determination of Urea Nitrogen.* Whole blood serum or plasma, properly buffered at a *p*H of 7.6, is acted upon by the enzyme urease, which quantitatively converts the urea present into ammonium carbonate. Ammonia is liberated by the addition of alkali and is aerated into a standard solution of hydrochloric acid. The excess acid is back titrated with standard alkali, giving the amount of ammonia formed and thus the concentration of urea nitrogen originally present.

$$\begin{matrix} & NH_2 & & \\ & \diagup & \text{urease} & \\ C & = & O & \xrightarrow[HOH]{} (NH_4)_2CO_3 \\ & \diagdown & & \\ & NH_2 & & \end{matrix}$$

$$(NH_4)_2CO_3 \xrightarrow[\text{aeration}]{OH^-} 2NH_3 + CO_2 + H_2O$$

$$2NH_3 + 2HCl \longrightarrow 2NH_4Cl$$

$$2HCl + 2NaOH \longrightarrow 2NaCl + 2H_2O$$

PROCEDURE. (**Warning**: Minute traces of Hg^{++} inhibit urease; therefore all glassware must be scrupulously cleaned. The urea tubes must never be used for any other purpose.)

Set up in a rack the tubes, bubblers, rubber connections, and so forth, as shown in the diagram (Fig. 19).

Place in tube *A*, which is permanent and can be used over and over again, approximately 15 ml. of 20 per cent sulfuric acid, 3 drops of methyl red and 1 ml. of antifoam mixture.

Place in tube *C*, 15.00 ml. of 0.01000 *N* hydrochloric acid, measuring it accurately from a burette or pipette.

Add approximately 1 ml. of antifoam mixture and 2 drops of methyl red as an indicator.

Add approximately 10 ml. of ammonia-free water, replace tube *C* in its proper place in the rack and stopper.

Transfer to tube *B* 3.00 ml. of blood, allowing the pipette to drain slowly. See that the blood does not touch the walls of the tube, but falls directly to the bottom of it.

Add 3 ml. of phosphate buffer solution and 1 or 2 ml. of urease suspension.

Stopper the tube immediately and mix the contents by gentle agitation. Allow to stand ten to fifteen minutes at room temperature.

* Marshall, *J. Biol. Chem.*, *15*, 487 (1913); Van Slyke and Cullen, *J. Biol. Chem.*, *19*, 211 (1914); Van Slyke and Cullen, *J. Biol. Chem.*, *24*, 117 (1916).

Make sure that all tubes are properly placed, the rubber connections rrect and gas-tight, and that the bubblers dip below the surface of the uids.

After ten to fifteen minutes, unstopper tube *B* and quickly add 1 ml. of tifoam mixture and 10 ml. of the saturated solution of potassium carbonate, owing the latter to run down the sides of the tube to form two layers.

Immediately stopper the tube and connect the outfit to the suction pump. Draw a current of air, gently for the first minute, then as rapidly as ssible without undue splashing and foaming.

Allow to aerate twenty-five to thirty minutes.

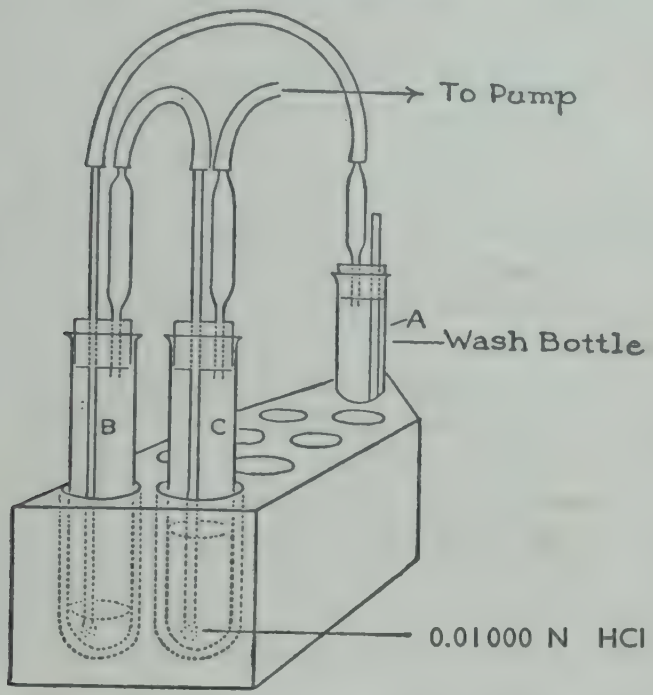

Fig. 19. Apparatus for urea nitrogen determination.

Turn off the suction, remove tube *C* and wash the liquid inside and on ıe walls of the bubbler into the tube.

Titrate the contents of tube *C* with standardized 0.01000 *N* sodium hyroxide.

Perform the procedure exactly as described, but use 3.00 ml. of ammonia-ee water instead of blood. This gives a blank analysis. (It is unnecessary in ıis case to wait the ten to fifteen minutes for enzyme action.)

Both the unknown and the blank should be done at the same time, with ıe same current of air, by connecting the pairs of tubes in series. (Many ımples may be run simultaneously in this fashion.)

Calculations. If the sodium hydroxide and hydrochloric acid used in ıis determination are not exactly 0.01000 *N*, convert them to equivalent olumes of 0.01000 *N* acid and alkali, using the relationship $\text{ml.}_1 \times N_1 = \text{ml.}_2 \times N_2$. Then $10\ (A - B - C)$ = mg. of urea per 100 ml. of blood.

$4.66\ (A - B - C)$ = mg. of urea N per 100 ml. of blood.

A = ml. of 0.01000 *N* acid used (usually 15 ml.).

B = ml. of 0.01000 *N* alkali used in titration.

C = value of $(A - B)$ found in blank determination.

REAGENTS:

20 PER CENT SULFURIC ACID. Mix 1 volume of concentrated sulfu acid with 4 volumes of water.

UREASE SUSPENSION. Any of the following preparations are satisfacto

A. Weigh 1 gm. of commercial urease preparation from jack bean me and add just sufficient water to form a paste. Mix well. Add more wat with stirring, so that the total quantity added is 9 ml. This suspensi should be freshly prepared each time.

B. Squibb's urease powder suspended as directed above.

C. Urease paper.

D. Urease—Glycerol Extract (Koch).—Place 37.5 gm. of Permutit in liter flask. Add 500 ml. of 2 per cent acetic acid. Shake well. Decant t liquid. Wash the Permutit in the flask with two 300 ml. portions of distill water, decanting the liquid each time. Add 75 gm. of jack bean meal the Permutit in the flask. Add 125 ml. of 0.001 *N* sulfuric acid and sha gently at ten minute intervals over the course of an hour. Add 375 ml. reagent grade glycerin. Mix well. Filter through a fluted filter paper into amber bottle. This preparation is stable for a year at room temperature a will keep indefinitely in the refrigerator. Five to 10 drops of it are sufficie for a determination.

PHOSPHATE BUFFER SOLUTION. Six grams of acid potassium phospha (KH_2PO_4) and 5 gm. of disodium phosphate ($Na_2HPO_4.12H_2O$) are di solved in water and made up to 1 liter.

SATURATED POTASSIUM CARBONATE SOLUTION. Nine hundred grams potassium carbonate are dissolved in a liter of water.

ANTIFOAM MIXTURE. Mix 1 volume of caprylic alcohol with 4 volume of 95 per cent ethyl alcohol.

0.1 PER CENT METHYL RED in 95 per cent alcohol.

0.01000 *N* HYDROCHLORIC ACID.

0.01000 *N* SODIUM HYDROXIDE.

(*a*) Using the urease method, what product other than ammonia coul be used for the estimation of urea?

(*b*) Illustrate the specificity of urease action.

(*c*) What method other than the urease method is considered a goo quantitative procedure?

(*d*) Name additional quantitative methods.

(*e*) How may a stable urease powder be prepared?

(*f*) What alternate method other than final titration of the ammonia ma be used?

(*g*) Can this method (*f*) be applied to urea in urine?

(*h*) Why is sulfuric acid placed in tube *A?*

(*i*) Why is methyl red added to tube *A?*

(*j*) Why is antifoam mixture added to tube *B?*

(*k*) Why cannot the potassium carbonate solution be added directly t the blood before urease is added?

(*l*) Why are two layers allowed to form between the blood and th potassium carbonate solution?

(*m*) Why is the suction gentle at first?

Experiment 8. Determination of Creatinine.* Protein-free, Folin-Wu blood filtrate is treated with an alkaline picrate solution, which turns red in the presence of creatinine. This color is compared with that developed by a standard solution. The reaction (the *Jaffe* reaction) is also caused by unknown substances in the blood filtrate other than creatinine and is not specific for creatinine. However, the amount of chromogenic substances called "creatinine" is of diagnostic significance.

PROCEDURE. Make a protein-free Folin-Wu filtrate, using blood or serum (see p. 79).

Transfer to a dry, labelled test tube, 10.00 ml. of the tungstic acid filtrate.

Into another similar test tube, transfer 5.00 ml. of the dilute standard creatinine solution, together with 15.00 ml. of water.

To the test tube containing the blood filtrate, add 5.00 ml. of the freshly prepared alkaline picrate solution.

To the test tube containing the dilute standard creatinine solution, add 10.00 ml. of the alkaline picrate solution.

Mix the solutions and allow them to stand for fifteen minutes.

Compare the solutions in a colorimeter† immediately thereafter.

It is essential that the depth of color in the standard and unknown be approximately the same. If it is not, repeat the procedure, using a more dilute or more concentrated standard creatinine solution.

When performing the determination photocolorimetrically, good results will be obtained with a green filter having a maximal transmission at 520 mμ.

Calculate the number of milligrams of creatinine per 100 ml. of blood or serum. If the visual colorimeter was used, refer to page 67 for the equation used. If the determination was performed photocolorimetrically, the appropriate relationships may be found on page 71, or a calibration curve may be used.

REAGENTS:

PICRIC ACID SOLUTION. Place 11.75 gm. of picric acid in a volumetric flask and dilute to 1 liter. Keep in a stoppered container to prevent evaporation.

10 PER CENT SODIUM HYDROXIDE SOLUTION.

ALKALINE PICRATE SOLUTION. Mix 5 volumes of picric acid solution with 1 volume of 10 per cent sodium hydroxide solution. This solution must be freshly made on the day used.

STOCK STANDARD 0.1 PER CENT SOLUTION OF CREATININE. Carefully weigh 1.000 gm. of purified creatinine and transfer it quantitatively to a liter volumetric flask, using 0.1 *N* hydrochloric acid for washing purposes. Make to mark with 0.1 *N* hydrochloric acid and mix. This solution contains 1.00 mg. of creatinine per milliliter. Transfer to a clean, dry, amber bottle and preserve with 2 ml. of toluene.

DILUTE STANDARD CREATININE. Transfer 3.00 ml. of the stock solution to a 500 ml. volumetric flask, add 50 ml. of 0.1 *N* hydrochloric acid, dilute

* Folin and Wu, *J. Biol. Chem., 38,* 81 (1919); Peters, *J. Biol. Chem., 146,* 179 (1942).

† By placing a Corning Glass Signal Purple No. 551 filter over the eyepiece of the visual colorimeter, comparisons may be made more easily.

to mark with water and mix. Transfer to a clean, dry bottle and preserve wi 0.5 ml. of toluene. This solution contains 0.0060 mg. of creatinine per mil liter.

(*a*) How may creatine be determined?

(*b*) Why must the solutions be compared within fifteen minutes?

(*c*) Why must both the standard and blood solutions be made up at t same time in all colorimetric procedures?

(*d*) Why should the solutions not be heated?

(*e*) How is total creatine-creatinine in the blood determined?

Experiment 9. The Determination of Uric Acid.* Protein-fre Folin-Wu blood filtrate is treated with a sodium cyanide-urea solution order to adjust the conditions so that the uric acid will have a maximu reducing effect. Upon the addition of phosphotungstic acid reagent, solub blue oxides of tungsten of lower valence states are formed. The intensity the color is a measure of the uric acid concentration.

PROCEDURE. Make a protein-free, Folin-Wu blood filtrate (see p. 79 Be sure to allow the mixture to stand for thirty minutes before filtering

Pipette 5.00 ml. of the tungstic acid filtrate into a test tube graduated 25 ml.

Into a similar tube, pipette 5.00 ml. of the dilute uric acid standard sol tion.

By means of a 10 ml. graduated cylinder, add to each tube 10 ml. c cyanide-urea reagent. **(Highly poisonous!)**

Whirl the tubes at an angle of 60 degrees so as to mix their content thoroughly.

Add 4.0 ml. of the uric acid reagent to each tube.

Again mix the tube contents thoroughly.

Allow the tubes to stand at room temperature for twenty minutes.

Dilute with distilled water to the 25 ml. mark.

Stopper the tubes and invert them several times in order to mix th contents.

Read in the colorimeter within five minutes after diluting.

When performing the determination photocolorimetrically, it is recom mended that but 1.00 ml. of protein-free blood filtrate be used. Four milli liters of distilled water are then added so as to keep the initial volume of flui at 5 ml. Good results will be obtained with a red filter having a maxima transmission at 640 mμ.

Caution. Those solutions which contain cyanide should be discarded b pouring them into the sink and then flushing them down the drain wit copious quantities of water. They should never be allowed to come into con

* Benedict, *J. Biol. Chem.*, *51*, 187 (1922); *54*, 233 (1922); Folin, *J. Biol. Chem.*, 5 179 (1930); *101*, 111 (1933).

† The whole blood used in the preparation of the protein-free filtrate for this de termination should contain the minimum amount of sodium or potassium oxalate a anticoagulant; otherwise the final colored solution may become turbid. The use of lithiu oxalate as an anticoagulant when uric acid is to be determined, largely avoids this diff culty.

act with acids, because the extremely poisonous hydrogen cyanide would hen be evolved.

Calculate the number of milligrams of uric acid per 100 ml. of blood. When using the visual colorimeter, refer to the equations on page 67. If a photoelectric colorimeter is used, the appropriate relationships may be found on page 71, or a calibration curve may be used.

REAGENTS:

CYANIDE-UREA REAGENT. Weigh into a 2 liter beaker, 75 gm. of C.P. sodium cyanide and dissolve the salt in about 700 ml. of water. Weigh 300 gm. of pure urea and dissolve it in the solution. Add 4 to 5 gm. of pure calcium oxide and stir for ten minutes. Allow to stand overnight and filter the next day. Add to the filtrate 2 gm. of lithium oxalate, shake for ten to fifteen minutes, and filter the solution into a clean bottle. (Keep in refrigerator.) **(Caution—sodium cyanide is extremely poisonous!)**

URIC ACID REAGENT. Transfer 200 gm. of sodium tungstate (which is molybdate-free) to a 750 ml. flask. Mix 65 ml. of 85 per cent syrupy phosphoric acid with 150 ml. of water, and add it slowly and with constant mixing to the sodium tungstate. Drop a few clean glass beads into the flask. Boil the solution gently for an hour, condensing the vapors by placing a funnel, holding a water-filled vessel, in the neck of the flask (or a reflux condenser will do). Remove the condenser and add a few milliliters of bromine water. Mix and then boil off the excess bromine in a hood. Add 5 to 10 gm. of sodium tungstate. Boil again for ten to fifteen minutes. Cool and decolorize as before by adding a little bromine water and then boiling off the excess. When cool, transfer to a 500 ml. volumetric flask. Dilute to mark, mix and store in an amber bottle.

LITHIUM OXALATE. Weigh 50 gm. of lithium carbonate into a 3 liter beaker. Add to it 85 gm. of crystalline oxalic acid. Pour into the mixture 1 liter of water, previously heated to 70°C., and stir vigorously until foaming ceases. Add 1 liter of 95 per cent alcohol. Stir and filter the solid on a Buchner funnel. Wash with a mixture of alcohol and water (1:1). Dry in air.

STOCK 0.1 PER CENT URIC ACID STANDARD SOLUTION. Weigh 1.000 gm. of uric acid and transfer it quantitatively to a 1 liter volumetric flask. To 0.6 gm. of lithium carbonate in a 250 ml. flask, add 150 ml. of water and shake until the salt is dissolved. Filter the solution. Heat the filtrate to 60°C. and warm the volumetric flask under running warm water. Pour the lithium carbonate solution into the flask, washing down the neck and sides with the solution. Shake the warm mixture for five minutes and quickly cool under running tap water (neglect any turbidity). Add 20 ml. of formalin (40 per cent formaldehyde) and half fill the flask with water. Add, by means of a pipette, 25 ml. of *N* sulfuric acid, while shaking the flask. Dilute to a liter, mix thoroughly and transfer to a clean, dry, well-stoppered amber bottle.

DILUTE URIC ACID STANDARD SOLUTION. By means of a pipette, transfer 1.00 ml. of the stock uric acid solution to a 250 ml. volumetric flask. Add 150 ml. of water, 15 ml. of dilute hydrochloric acid (1 volume of concentrated acid to 9 of water) and dilute to mark. Transfer to a clean, dry bottle. This solution, containing 0.0040 mg. of uric acid per milliliter, should be freshly prepared every week.

(*a*) What substance in blood seriously interferes with this determinati
(*b*) What other method may be applied to urine?
(*c*) Why is method (*b*) not applicable to blood?
(*d*) Why is cyanide-urea added?
(*e*) Why are tubes allowed to stand for twenty minutes?
(*f*) What gas is formed when the uric acid reagent is added to both tu
containing cyanide-urea?

Experiment 10. The Determination of Cholesterol.* The lipids
blood or serum are extracted with an alcohol-ether mixture; at the same ti
the proteins are precipitated. An aliquot of the extract is evaporated to d
ness; the residue is then extracted with chloroform. Taking advantage of
Liebermann-Burchard reaction, acetic anhydride and sulfuric acid are add
to the chloroform solution, giving rise to a blue-green color, the depth
which is a measure of the amount of total cholesterol present (free choleste
plus cholesterol esters).

EXTRACTION. Pour about 20 ml. of Bloor's alcohol-ether mixture int
dry 25 ml. volumetric flask.

Add slowly, from a pipette, 1.00 ml. of whole blood, serum or plasm
keeping the contents of the flask in constant motion to prevent clumpi
of the precipitate which forms.

Dilute to the 25 ml. mark with Bloor's mixture. Stopper immediately a
shake vigorously.

Allow to stand for at least one hour at room temperature, shaking occ
sionally.

Insert a dry, fluted filter paper in a dry funnel.

Pour the contents of the volumetric flask onto the filter paper, immediate
covering the funnel with a watch glass.

Collect the filtrate in a dry 50 ml. Erlenmeyer flask. (If both total chol
terol and cholesterol esters are to be determined, all quantities in this pr
cedure may be doubled. The filtrate may be used for both determinations

PROCEDURE. As soon as 12 to 15 ml. of the filtrate have been collecte
transfer 10.00 ml. of it to a 50 ml. dry beaker.

Evaporate almost to dryness on a steam bath,† completing the tot
evaporation of the solvent at room temperature. (A gentle current of dry a
may be used to blow off the last traces of solvent.)

By means of a dry medicine dropper, add about 3 ml. of chloroform
the residue, washing down the sides of the beaker during the addition.

Heat the beaker until the chloroform is warm, but do not boil.

Carefully decant the cholesterol extract into a dry, 10 ml. glass-stoppere
graduated cylinder.

Repeat the extraction of the residue with another 3 ml. of chloroform, a
decant into the same cylinder. Repeat a third time.

* Bloor, Pelkan and Allen, *J. Biol. Chem.*, *52*, 191 (1922); Sackett, *J. Biol. Chem.*,
203 (1915); Bloor, *J. Biol. Chem.*, *77*, 53 (1928); Schoenheimer and Sperry, *J. Bi*
Chem., *106*, 745 (1934).

† Presence of water interferes with the color.

Allow the solution in the cylinder to reach room temperature and then add chloroform to the 10 ml. mark. Stopper immediately thereafter.

Pipette 5.00 ml. of the dilute standard cholesterol solution into a second 10 ml. glass-stoppered graduated cylinder, which must be dry.

Dilute to the 10 ml. mark with chloroform and stopper immediately.

Add to both cylinders 2.0 ml. of acetic anhydride and 0.1 ml. of concentrated sulfuric acid.

Immediately replace the stoppers in the cylinders and invert them several times.

Place the cylinders in a dark place, preferably at 25° to 26°C., for fifteen minutes.

Immediately after that time has elapsed, read the solutions in the colorimeter.

When performing the determination photocolorimetrically, a red filter with maximal transmission at 625 mμ. will give good results.

Calculate the number of milligrams of cholesterol per 100 ml. of serum. When using the visual colorimeter, refer to the equations on page 67. If a photoelectric colorimeter is used, the appropriate relationships may be found on page 71, or a calibration curve may be used.

REAGENTS:

BLOOR'S MIXTURE. Three parts of 95 per cent ethyl alcohol and 1 part of ether. Both the ether and alcohol must have been previously distilled.

CHLOROFORM. Redistill, water-free.

ACETIC ANHYDRIDE, C.P.

SULFURIC ACID, concentrated C.P.

STOCK 0.1 PER CENT CHOLESTEROL SOLUTION IN CHLOROFORM. Carefully weigh out 0.100 gm. of recrystallized pure cholesterol and transfer to a 100 ml. clean, dry volumetric flask. Add chloroform up to mark, stopper and shake to dissolve the solid. Transfer immediately to a well-stoppered dry bottle.

DILUTE STANDARD CHOLESTEROL SOLUTION IN CHLOROFORM. Transfer 1.00 ml. of the stock cholesterol solution to a dry 10 ml. volumetric flask. Make to mark with chloroform. Keep stoppered. Make up freshly each time. This solution contains 0.10 mg. of cholesterol per milliliter.

(*a*) What are good solvents for lipids?
(*b*) What gravimetric method may be used to determine cholesterol?
(*c*) How can we differentiate free and combined cholesterol?
(*d*) What form of cholesterol does the colorimetric procedure determine?
(*e*) Is the colorimetric method specific for cholesterol?

Experiment 11. The Determination of Cholesterol Esters. Since little cholesterol ester is found in red cells, serum or plasma is the preferred medium. It is extracted with alcohol-ether mixture to remove the lipids and to precipitate the proteins. The free cholesterol in the extract is precipitated as the insoluble cholesterol-digitonide by the addition of digitonin. After evaporating to dryness, the cholesterol esters are extracted with petroleum ether and determined by the *Liebermann-Burchard* reaction.

PROCEDURE. Prepare an alcohol-ether filtrate of the serum or plasm as directed under *Extraction*, page 98.

Transfer 15.00 ml. of the alcohol-ether filtrate to a dry 50 ml. beaker.

Add 1 ml. of 1 per cent alcoholic digitonin solution. Mix by gent rotating the beaker.

Evaporate almost to dryness on a steam bath and complete the tot evaporation of the solvent at room temperature. (A gentle current of dry a may be used to blow off the last traces of solvent.)

Add 15 ml. of petroleum ether.

Cover with a watch glass, place in a 60° to 70°C. water bath. (Be caref of flames!)

Boil gently until about half the liquid remains.

Cool and then carefully decant the ether extract into a dry 50 ml. beake

Repeat the extraction and decantation with two succeeding portions c 7 to 8 ml. of petroleum ether.

Evaporate the combined extracts to dryness. (The oily film remaining i the beaker is the cholesterol ester.)

Extract the residue with 3 ml. portions of chloroform and continue th procedure exactly as described under The Determination of Cholesterol page 98, beginning with, "By means of a dry medicine dropper. . . ."

REAGENTS:

PETROLEUM ETHER. Reagent grade (B.P. 30° to 60°C.).

1 PER CENT DIGITONIN SOLUTION. Dissolve 1 gm. of digitonin in 100 ml. of 80 per cent ethyl alcohol. Cool and filter into an amber bottle.

OTHER REAGENTS. Refer to Experiment 10, under *Reagents.*

Experiment 12. The Determination of Sulfonamides.* Blood is laked by the addition of water, and the proteins are precipitated with trichloracetic acid. The acid filtrate so obtained is treated with a sodium nitrite solution. Those sulfonamides having a free amino group on the benzene ring are diazotized. Excess nitrite ion, which interferes with the subsequent development of color, is destroyed by the addition of ammonium sulfamate. The diazo compound is coupled with N-(1-naphthyl) ethylenediamine dihydrochloride to form a stable, soluble dye. The intensity of the purplish-red color produced is proportional to the concentration of the free sulfa compound.

PROCEDURE. Pipette 31.00 ml. of water into a dry 125 ml. Erlenmeyer flask.

Add 1.00 ml. of oxalated blood, allowing the pipette to drain slowly.

Mix thoroughly and allow to stand several minutes to insure complete laking.

Add 8.00 ml. of 15 per cent, freshly prepared trichloracetic acid.

Stopper the flask and shake it vigorously.

Filter through a dry filter paper into a dry flask, pouring back the first few drops for refiltration if necessary.

* Marshall and Litchfield, *Science, 88,* 285 (1938); Bratton and Marshall, *J. Biol. Chem., 128,* 537 (1939).

Pipette 10.00 ml. of the water-clear filtrate into a test tube graduated at ml.

Into a second similar tube, pipette 5.00 ml. of the dilute standard sulfonamide solution and 2 ml. of 15 per cent trichloracetic acid.

****Add 0.5 ml. of 0.1 per cent sodium nitrite solution to each tube.

Mix thoroughly and allow to stand for four minutes to obtain complete azotization.

Add 0.5 ml. of 0.5 per cent ammonium sulfamate solution to each tube.

Mix thoroughly and allow to stand for three minutes.

Add 0.5 ml. of 0.1 per cent N-(1-naphthyl) ethylenediamine dihydrochloride solution and mix thoroughly once again.

Allow the color to develop for at least one minute.

Dilute to the 20 ml. mark with distilled water, stopper the tubes, and mix by inverting them several times.

Compare the solutions in the colorimeter within thirty minutes of the final dilution.

Some of the total sulfonamide which may be present in blood—and practically all of that in the urine—is in a combined form. The liver acetylates the amino group. Such conjugated sulfonamides do not diazotize or yield a dye by the above reactions.

In order to determine the total sulfonamide concentration in blood, 10.00 ml. of the protein-free filtrate are placed in a tube graduated at 20 ml. and then heated in an actively boiling water bath for forty-five to sixty minutes with 0.5 ml. of 4 *M* hydrochloric acid. This hydrolyzes the conjugated drug. The solution is cooled and then treated as in the determination of free sulfonamides (see above). The difference between the values of the free and total sulfonamides gives the concentration of conjugated drug.

Sulfonamides in urine may be determined in a similar manner upon appropriate dilution.

CALIBRATION CURVE. Place six large test tubes graduated at 20 ml. in a rack. Pipette into them the quantities of dilute standard sulfonamide solution and water listed in the table below:

Tube Number	*Ml. of Dilute Standard Sulfonamide Solution*	*Ml. of Water*	*Mg. Per Cent*
1	2.00	10.00	4
2	4.00	8.00	8
3	6.00	6.00	12
4	8.00	4.00	16
5	10.00	2.00	20
6	12.00	0.00	24

Add to each 2 ml. of 15 per cent trichloracetic acid.

Continue exactly as the above for the unknown from the asterisks on.

Read in a photoelectric colorimeter, using the appropriate filter. One with maximal transmission in the green at 545 mμ. will give good results.

Plot the data on linear or semilog graph paper.

Sulfa drugs such as sulfanilamide, sulfapyridine, sulfathiazole, sulfa-

diazine, sulfaguanidine, sulfamerazine, and the like are all determined in t above manner. In each case, however, standards of the particular dr should be used.

REAGENTS:

STOCK STANDARD SULFONAMIDE SOLUTION. Weigh 0.1000 gm. of pu dry sulfonamide and transfer it to a 1 liter volumetric flask with a lit distilled water. Add a few drops of 10 per cent sodium hydroxide or 2 sulfuric acid to dissolve the solid. Neutralize approximately. Dilute to ma with distilled water. Mix thoroughly. Transfer to a dry, amber bottle. Th solution contains 0.10 mg. of sulfonamide per milliliter, and is stable f several months if kept refrigerated.

DILUTE STANDARD SULFONAMIDE SOLUTION. Pipette 5.00 ml. of th stock standard sulfonamide solution into a 100 ml. volumetric flask. Dilu to mark with distilled water. Mix thoroughly. This solution is stable for b a few days. It contains 0.0050 mg. of sulfonamide per milliliter.

TRICHLORACETIC ACID, 15 PER CENT. Weigh out quickly in a sma beaker counterpoised on the pan of a scale, about 18 gm. of pure trichlo acetic acid. (*This must be done rapidly, since the material is very hygro scopic; and cautiously, because it produces painful burns if it comes in con tact with the skin!*) Add 82 ml. of water. Dissolve, mix and transfer to clean bottle. Make up freshly once a week. Store in the refrigerator.

SODIUM NITRITE, 0.1 PER CENT. Dissolve 1 gm. of sodium nitrite i distilled water and make up to 1 liter in a volumetric flask. This reagen will not keep for more than a day.

N-(1-NAPHTHYL) ETHYLENEDIAMINE DIHYDROCHLORIDE, 0.1 PER CENT Dissolve 100 mg. of the compound in distilled water and dilute to 100 ml in a volumetric flask. Keep in a glass-stoppered, dark bottle.

AMMONIUM SULFAMATE, 0.5 PER CENT. Dissolve 0.5 gm. of crystalline ammonium sulfamate in distilled water and dilute to 100 ml. in a volumetric flask.

HYDROCHLORIC ACID, 4 M. Dilute 33 ml. of concentrated hydrochloric acid to 100 ml.

Experiment 13. The Determination of Phosphorus (Inorganic Phos phates).* The phosphates in a protein-free blood filtrate are mixed with excess ammonium molybdate to form ammonium phosphomolybdate. 1,2,4-aminonaphtholsulfonic acid made up in a sulfite-bisulfite solution is added to reduce the hexavalent molybdenum of phosphomolybdic acid to blue-colored reduction products. The reagent has a negligible effect on the hexavalent molybdenum of molybdic acid. The color thus produced is a measure of the amount of phosphomolybdic acid formed and hence of the concentration of phosphate ion originally present.

PROCEDURE. Pipette into a dry 50 ml. Erlenmeyer flask, 8.00 ml. of a 10 per cent trichloracetic acid solution.

Rotate the flask gently and slowly add, from a pipette, 2.00 ml. of whole blood (serum or plasma), keeping the flask constantly in motion.

* Bell and Doisy, *J. Biol. Chem.*, *44*, 55 (1920); Briggs, *J. Biol. Chem.*, *53*, 13 (1922); *59*, 255 (1924); Fiske and Subbarow, *J. Biol. Chem.*, *66*, 375 (1015); Youngburg and Youngburg, *J. Lab. and Clin. Med.*, *16*, 158 (1930); Kuttner and Cohen, *J. Biol. Chem.*, *75*, 517 (1927); Kuttner and Lichtenstein, *J. Biol. Chem.*, *86*, 671 (1930).

Stopper the flask and then shake it vigorously.

Place a dry, folded piece of filter paper in a dry funnel, and beneath it a dry 50 ml. Erlenmeyer flask.

Filter the mixture and cover the funnel with a watch glass.

Transfer 5.00 ml. of the filtrate to a 10 ml. volumetric flask and stopper it.

Transfer 5.00 ml. of the standard phosphate solution to a 100 ml. volumetric flask. Add approximately 60 ml. of water.

To the unknown add 1.00 ml. of 2.5 per cent ammonium molybdate solution in 3 *N* sulfuric acid and mix.

To the standard add 10 ml. of ammonium molybdate solution in 5 *N* sulfuric acid and mix.

To the unknown add, by means of a 1 ml. Mohr pipette, exactly 0.4 ml. of the sulfonic acid reagent and mix.

To the standard add 4.00 ml. of the sulfonic acid reagent and mix.

Dilute each solution to mark with water.

Stopper each flask, mix the contents thoroughly, and allow to stand for eight minutes.

Compare in a colorimeter immediately thereafter.

When performing the determination photocolorimetrically, good results will be obtained with a red filter having a maximal transmission at 640 millimicrons.

Calculate the number of milligrams of phosphorus per 100 ml. of blood or serum. If the visual colorimeter was used, refer to page 67 for the equation. If the determination was performed photocolorimetrically, the appropriate relationships may be found on page 71, or a calibration curve may be used.

REAGENTS:

10 PER CENT TRICHLORACETIC ACID. Weigh out quickly in a small beaker counterpoised on the pan of a scale, about 12 gm. of pure trichloracetic acid. (See precautions for preparation, p. 102). Add 88 ml. of water. Dissolve, mix, and transfer to a clean bottle. Make up freshly once a week. Store in the refrigerator.

2.5 PER CENT AMMONIUM MOLYBDATE SOLUTION IN 5 *N* SULFURIC ACID. Weigh out 25 gm. of ammonium molybdate and dissolve in 200 ml. of water. Transfer it quantitatively to a 1 liter volumetric flask containing 500 ml. of 10 *N* sulfuric acid, rinsing with water. Dilute to mark with water, mix, and transfer to a clean, dry bottle.

2.5 PER CENT AMMONIUM MOLYBDATE SOLUTION IN 3 *N* SULFURIC ACID. Prepare as before, but place only 300 ml. of 10 *N* sulfuric acid in the volumetric flask.

0.25 PER CENT 1,2,4-AMINONAPHTHOLSULFONIC ACID. Place about 90 ml. of water, which has been heated to 95°C., in a 100 ml. volumetric flask, which is then kept warm by placing on a steam bath. Weigh 15 gm. of sodium bisulfite and dissolve it in the hot water in the volumetric flask. Weigh 0.25 gm. of 1,2,4-aminonaphtholsulfonic acid and transfer it to the flask, shaking to suspend it uniformly throughout the liquid. Add to the suspension 1 gm. of sodium sulfite and shake. All the solids should dissolve. If not, add small amounts of solid sodium sulfite until complete solution is obtained. Cool the flask and contents to room temperature by holding it under

cold, running tap water. Dilute to mark and filter. This solution must b made up freshly every week.

STANDARD PHOSPHATE SOLUTION. Weigh 0.3509 gm. of pure mon potassium phosphate and quantitatively transfer it to a 1 liter volumetri flask. Add about 500 ml. of water and 10 ml. of 10 *N* sulfuric acid. Mix an dilute to mark. Transfer to a clean, dry, amber bottle. This standard contain 0.080 mg. of phosphorus per milliliter.

(*a*) What gravimetric methods may be used?

(*b*) What volumetric methods may be used?

(*c*) Why cannot tungstic acid be used to precipitate proteins in phosphorus determination?

(*d*) What are some other agents used to reduce phosphomolybdate?

(*e*) Why is it necessary to use two ammonium molybdate solutions?

(*f*) Why are sodium bisulfite and sodium sulfite added to the 1,2,4 aminonaphtholsulfonic acid?

Experiment 14. The Determination of Serum Alkaline Phosphatase.* Serum or plasma contains an enzyme capable of catalyzing the hydrolysis of certain organic phosphates. The optimum activity of this enzyme is at a *p*H of about 8.6.

The phosphatase content is determined by estimating the amount of the product of enzymatic activity formed in a certain time, under fixed conditions of *p*H, temperature, and salt content. One of the most widely used units of phosphatase activity is that of Bodansky, which is defined as the amount of enzyme which liberates 1 mg. of phosphorus, as phosphate ion, during the first hour of incubation at 37°C. and at *p*H 8.6, from the substrate, sodium beta-glycerophosphate, the hydrolysis not exceeding 10 per cent of the substrate.

The serum is incubated with a buffered glycerophosphate solution. The phosphorus (as the phosphate ion) released by the phosphatase present is obtained by determining the difference between the phosphorus content after incubation and the phosphorus content of the serum. The phosphorus is determined in the usual manner (p. 102).

PROCEDURE. Insert a pipette through the petroleum ether layer into the buffered glycerophosphate solution, holding the finger over the upper end of the pipette to prevent any of the petroleum ether from getting into the pipette. Transfer about 7 ml. of the glycerophosphate into a dry test tube, avoiding aeration of the substrate, and place in a water bath at 37°C. for ten minutes in order to reach that temperature.

Transfer a freshly drawn sample of blood to a clean, dry centrifuge tube. Allow to stand until the clot which forms contracts, and the clear serum separates. Gently loosen the clot with a stiff wire.

Centrifuge a sample of unhemolyzed blood. Draw off the clear serum by means of a pipette, taking care not to mix red cells with the serum.

Keep the clear serum at 37°C. for several minutes.

Transfer 5.00 ml. of the glycerophosphate solution to a small test tube, and place in the water bath at 37°C. for a few minutes.

* Bodansky, *J. Biol. Chem.*, *101*, 93 (1933); Roe and Whitmore, *Am. J. Clin. Path.*, *8*, 233 (1938).

Add 0.50 ml. of the serum, mix, and stopper loosely with a rubber stopper. Replace the test tube and its contents in the water bath, and allow to and at 37°C. for exactly one hour from the time of the addition of the erum to the glycerophosphate solution.

In the meantime, transfer 2.00 ml. of serum to a dry 50 ml. Erlenmeyer ask and start the determination of inorganic phosphorus (p. 102).

Exactly one hour after the addition of the serum to the buffered gly-erophosphate solution, remove the test tube, cool immediately in ice water, nd add 4.50 ml. of 15 per cent trichloracetic acid while rotating the tube ently.

Shake the tube well, and centrifuge. Transfer 5.00 ml. of the supernatant) a 10 ml. volumetric flask and determine the inorganic phosphorus present p. 102).

Calculate the number of alkaline phosphatase units per 100 ml. of serum. 'his is equal to the milligrams per cent of phosphorus determined in the ncubated sample of serum (due to the original phosphorus content plus the hosphorus hydrolyzed from organic phosphates under the influence of the hosphatase) minus the milligrams per cent of phosphorus in the original erum.

Reagents:

Buffered Glycerophosphate Substrate Solution. Dissolve 2.5 gm. f sodium beta-glycerophosphate and 2.12 gm. of monosodium diethyl-arbiturate in distilled water, and make up to mark in a 500 ml. volumetric lask. Preserve the substrate in the refrigerator in glass stoppered bottles 100 or 250 ml.) under a layer of washed petroleum ether (B.P. 30° to 6°C.) about 3 cm. thick. The reagent should be freshly prepared every two nonths.

Trichloracetic Acid, 15 Per Cent. Eighteen grams of pure tri-hloracetic acid are added to 82 ml. of distilled water. (See precautions for reparation, p. 102.)

For other reagents, see the determination of inorganic phosphorus (p. 103).

(*a*) Write the structural formula of sodium beta-glycerophosphate.

(*b*) Why is it important to incubate for exactly one hour?

(*c*) Does the *p*H of the mixture change during incubation? Why?

(*d*) Why is the substrate covered with petroleum ether and kept in the refrigerator?

(*e*) What is the probable function of alkaline phos-phatase in the body?

(*f*) What are normal phosphatase values for (1) children, (2) adults?

(*g*) In what diseases may the phosphatase values be high?

(*h*) Name and describe two other units of phos-phatase activity besides the Bodansky unit.

(*i*) Why must unhemolyzed blood or serum not ontaining red cells be used in the phosphorus deter-nination?

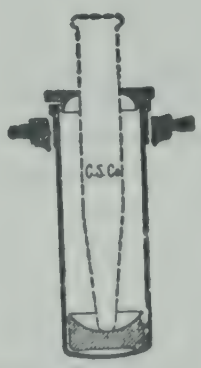

Fig. 20. Centrifuge tube fitted into a trunnion.

Experiment 15. The Determination of Calcium.* Serum or plasm is treated with ammonium oxalate, precipitating the calcium as calciu oxalate. The washed precipitate is dissolved in sulfuric acid and titrated wi a standardized potassium permanganate solution.

$$Ca^{++} + C_2O_4^{=} \longrightarrow CaC_2O_4$$
$$CaC_2O_4 + H_2SO_4 \longrightarrow H_2C_2O_4 + CaSO_4$$
$$5H_2C_2O_4 + 2KMnO_4 + 3H_2SO_4 \longrightarrow K_2SO_4 + 2MnSO_4 + 10CO + 8H_2$$

USE OF THE CENTRIFUGE. Transfer the material to be centrifuged to dry conical 15 ml. centrifuge tube.

Place the tube in a brass centrifuge cup which is fitted into a trunnio (Fig. 20). (See that there is a rubber cushion in the bottom of the brass cup.

Add water to a second centrifuge tube until its volume is approximatel the same as that of the liquid in the first tube. Place the tube in another bras cup fitted with a trunnion.

Put the two assemblies on the pans of a scale. With a pipette or was bottle, add water to one of the brass cups until the assemblies exactly balance

Place the assemblies on opposite arms of the centrifuge head. (Severa pairs may be centrifuged at the same time, depending on the size of the head Each individual pair must be balanced. The balanced assemblies must be placed opposite each other.)

Close the cover of the centrifuge. Slowly bring up the rheostat until the desired speed is attained. (If the centrifuge, at high speed, rattles or rumbles, stop it and rebalance the cups.)

Allow the centrifuge to run at high speed for fifteen to twenty minutes to separate red cells from plasma.

Rapidly bring the rheostat to the "Off" position. Allow the centrifuge to slow down to a stop. (Do not apply the brake or open the cover until the head ceases to rotate.)

After a sample of blood has been centrifuged, the plasma may be withdrawn without disturbing the cells by the careful use of a pipette which has a piece of rubber tubing attached to the upper end. Good control of the suction may be accomplished by squeezing the rubber tube with the fingers.

PROCEDURE. Obtain a sample of heparinized blood. (Blood treated with fluoride, oxalate or citrate cannot be used. The serum obtained from a sample of clotted blood or from defibrinated blood may be used.)

Centrifuge the blood and withdraw the plasma, taking care not to dilute it.

Transfer 2.00 ml. of the plasma (or serum) to a scrupulously clean 15 ml. conical centrifuge tube. (The tubes should be heated at 100°C. in sulfuric acid-dichromate "cleaning mixture" before use. They should then be rinsed with copious quantities of tap and then distilled water.)

Add to the tube 2 ml. of water and 1 ml. of 4 per cent ammonium oxalate solution, allowing the solutions to drop directly into the plasma and not touching the sides of the tube.

Mix the contents by holding the tube at the mouth with the thumb and

* Kramer and Tisdall, *J. Biol. Chem.*, *47*, 475 (1921); Clark, *Ibid.*, *39*, 487 (1921); Tisdall, *Ibid.*, *56*, 439 (1923); Clark and Collip, *Ibid.*, *63*, 461 (1925).

ndex finger and imparting a circular movement to the contents by tapping ne lower end of the tube. (When the material at the apex of the tube has been mixed, the process has been completed.)

Allow to stand thirty minutes to one hour.

Thoroughly mix the contents.

Centrifuge for seven minutes at high speed, taking all precautions.

Carefully pour off the supernatant fluid from the tube without disturbing.

While the tube is still inverted, let it drain in a rack for five minutes, esting the mouth of the tube on a pad of filter paper.

Wipe the mouth of the tube with a piece of filter paper.

Wash down the sides of the tube with 3 ml. of 2 per cent ammonia water.

Stir the precipitate in the wash ammonia in the manner described above. The rotary motion should break up the clump of precipitate.)

Centrifuge the suspension for seven minutes at high speed, pour off the upernatant liquid, and allow to drain for five minutes, as before.

Add 2 ml. of approximately *N* sulfuric acid, blowing it from a pipette lirectly upon the precipitate so as to break up the mat and facilitate its olution. (The precipitate should dissolve.)

Place the tube in a boiling water bath for about one minute.

Keeping the tube at a temperature of 70° to 75°C. by immersing it in ıot water, titrate with approximately 0.005 *N* potassium permanganate to a lefinite pink color persisting for one minute.

Standardize the potassium permanganate solution by measuring from he burette 10.00 ml. of standard 0.005000 *N* sodium oxalate solution, adding 2 ml. of *N* sulfuric acid, heating to 70° to 75°C., and titrating to a definite ɔink color with the potassium permanganate solution.

A blank determination is made by repeating the procedure exactly as ıbove, substituting 2 ml. of water for the 2 ml. of plasma.

CALCULATIONS:

$$10 \times \frac{N}{P} \times \frac{(A - B)}{v} = \text{mg. Ca per 100 ml. of plasma}$$

N = ml. of 0.005000 N sodium oxalate used in the standardization of the permanganate (10.00 ml.)

P = ml. of approximately 0.005 N permanganate used to titrate the above volume of 0.00500 N sodium oxalate solution

A = ml. of approximately 0.005 N permanganate used in the titration of the unknown

B = ml. of approximately 0.005 N permanganate used in the titration of the blank

v = ml. of plasma used

REAGENTS:

AMMONIUM OXALATE. Saturated aqueous solution (about 4 per cent).

2 PER CENT AMMONIA WATER. Mix 1 volume of concentrated ammonium hydroxide with 50 volumes of water.

APPROXIMATELY *N* SULFURIC ACID. Add 20 ml. of concentrated sulfuric ıcid (36 *N*) to 700 ml. of water. Mix.

STOCK 0.1 *N* POTASSIUM PERMANGANATE. Weigh 3.16 gm. of potassium

permanganate and transfer to a 1 liter volumetric flask. Add about 850 m of water. Dissolve the solid completely. Make up to mark. Mix and trans to a clean, dry, glass-stoppered, amber bottle. This solution becomes weak on standing.

APPROXIMATELY 0.005 *N* POTASSIUM PERMANGANATE. Pipette 50 ml. the stock 0.1 *N* solution into a liter volumetric flask and dilute to mar Mix and transfer to a clean, dry, amber bottle. This solution must standardized by titration with 0.005000 *N* sodium oxalate each time befo use.

STOCK 0.1000 *N* SODIUM OXALATE. Carefully weigh 6.700 gm. of C. anhydrous sodium oxalate. Quantitatively transfer the solid to a liter vol metric flask. Dissolve the solid in about 700 ml. of water. Add 105 ml. of sulfuric acid. Make up to mark with distilled water. Mix and transfer to clean, dry, amber bottle. The solution is stable.

0.005000 *N* SODIUM OXALATE. Using a clean, dry pipette, transf 50.00 ml. of the stock 0.1000 *N* solution into a clean 1 liter volumetric flas and dilute to mark. Mix and transfer to a clean, dry, amber bottle.

(*a*) Discuss several methods of determining calcium oxalate other tha titration with permanganate.

(*b*) What substance might interfere in the permanganate method?

(*c*) Why cannot tungstic acid be used for precipitating proteins in calcium determination?

(*d*) How does use of anticoagulants effect the determination of calcium

(*e*) What is the purpose of the rubber cushion in the centrifuge?

(*f*) Why must two balanced centrifuge tubes be used?

(*g*) Why must the trunnions on the cups be in the same position?

(*h*) Why is the rheostat on the centrifuge brought up slowly?

(*i*) What is the danger in stopping the centrifuge before it would nor mally come to a stop?

(*j*) Can Ca^{++} be determined in whole blood?

(*k*) Why is 2 per cent ammonia water used instead of water for washin the precipitate?

(*l*) Why must the permanganate solution be standardized?

(*m*) Why must the calcium oxalate be titrated with permanganate whil hot?

(*n*) Why must the oxalate be titrated in acid solution?

Experiment 16. The Determination of Chlorides.* Serum or plasm is shaken for about 40 seconds with powdered, chloride-free, silver iodat There is an exchange of the iodate of the sparingly soluble silver iodate fo the chloride ion in the serum to yield iodate ion and the very insoluble silve chloride. Under the conditions of the method the reaction is 99.7 per cen complete. Simultaneously the serum proteins are precipitated by the additio of sodium tungstate in phosphoric acid solution. The clear filtrate from th above mixture is protein-free and contains the liberated iodate ion in concentration equivalent to the original chloride ion. The solubility of th

* Sendroy, *J. Biol. Chem.*, *130*, 605 (1939); Van Slyke and Hiller, *J. Biol. Chem.*, *16* 107 (1947).

solid silver iodate is repressed to a negligible value by the iodate ion in the solution. Sodium iodide is added to the acid solution to convert the iodate ion to iodine. The iodine is titrated with a standard thiosulfate solution in the usual manner.

$$AgIO_3 + Cl^- \longrightarrow AgCl\downarrow + IO_3^-$$
$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$
$$I_2 + S_2O_3^= \longrightarrow 2I^- + S_4O_6^=$$

In blood as it is drawn, only a few of the dissolved substances have identical concentrations in the plasma and cells, due to the "Chloride Shift." Chlorides are almost twice as concentrated in the plasma as in cells. Soon after the blood is exposed to air, it loses carbon dioxide and the constituents of the two phases diffuse into one another. After a time an equilibrium is reached in which the non-colloidal blood constituents are uniformly distributed in the medium. From a clinical point of view, the plasma or serum chlorides have more significance than the total or blood chlorides. It is, therefore, essential to prevent the chlorides from diffusing from the plasma to the red cells after the blood is drawn and until the serum is separated for analysis, or the results will not indicate serum concentrations in vivo. It is advisable to draw the blood under oil in order to delay the escape of carbon dioxide, and then to separate the serum from the cells as soon as possible.

PROCEDURE. Place about 3 to 5 ml. of mineral oil in a dry, clean centrifuge tube.

Obtain a blood specimen with a dry syringe and a minimum of stasis.

Transfer the blood to the centrifuge tube, under the layer of oil.

Stopper the tube and allow it to stand until a clot forms and the clear serum separates.

Centrifuge the tube. (See p. 106.)

Holding the finger tightly over the upper opening of a clean, dry 5 ml. pipette, plunge it through the layer of oil in the centrifuge tube and then draw off the clear serum. Transfer it to another clean, dry tube.

Transfer 1.00 ml. of the serum to a 125 ml. Erlenmeyer flask.

Pipette 25.00 ml. of the tungstate-phosphoric acid solution into the flask.

By means of a small glass spoon or a horn spatula, add from 0.3 to 0.5 gm. of silver iodate powder to the flask.

Stopper the flask with a clean stopper and shake the mixture *vigorously* for 40 seconds.

Have ready a dry, folded filter paper in a dry funnel, beneath which is a dry 125 ml. Erlenmeyer flask.

Pour a little of the above mixture onto the double portion of the filter paper. When the whole paper has become wet, pour the remainder of the mixture into the funnel.

Cover the funnel with a watch glass and collect the protein-free filtrate. (It should be perfectly clear and colorless. If the first small amount of the filtrate is cloudy, it should be refiltered.)

(Instead of the filtration outlined in the above steps, the serum, tungstate-phosphoric acid and silver iodate, may be placed in a clean, dry, 50 ml.

centrifuge tube, which is then shaken and finally capped and centrifug to separate a clear supernatant fluid.)

Pipette 10.00 ml. of the filtrate into a small Erlenmeyer flask. Add fr 0.5 to 1.0 ml. of sodium iodide solution.

Immediately after the addition of the sodium iodide, titrate the soluti with 0.02308 *N* thiosulfate solution delivered from a 25 ml. burette. (T thiosulfate solution should be delivered dropwise—not in a stream—un the color of the solution in the flask becomes a pale yellow. Two drops starch solution are then added. The titration is continued, a drop at a tim until the blue solution suddenly turns colorless. The end-point can be locat within 0.01 ml. of the thiosulfate solution. No time should be lost after t iodine is liberated, as it volatilizes rapidly—about 2 per cent per hour.)

BLANK. It is advisable to run a blank in order to check the enti technic—including the accuracy of the pipettes used, the purity of t sodium tungstate, silver iodate and other reagents. This is best done b using 1.00 ml. of 0.1000 *N* HCl, prepared from constant boiling HC instead of the 1.00 ml. of the serum; and running through the procedur Ten ml. of the 0.02308 *N* thiosulfate should be required for the titration. more or less is used, the difference is subtracted or added to the amou used in the serum analysis as a blank correction.

CALCULATIONS:

Milliequivalents of chloride per liter of serum = 10T

Milligrams of NaCl per 100 ml. of serum = 58.5T

Milligrams of chloride per 100 ml. of serum = 34.45T

where T is the number of ml. of 0.02308 *N* thiosulfate solution used in th titration.

REAGENTS:

TUNGSTATE-PHOSPHORIC ACID SOLUTION. Dissolve 6 gm. of A.C.S chloride-free, sodium tungstate ($Na_2WO_4.2H_2O$) in 1 liter of distilled wate and add 10 ml. of concentrated phosphoric acid (sp. gr. 1.72). Mix wel and transfer to a clean bottle.

SILVER IODATE POWDER. C.P. Especially prepare for chloride analysis

SODIUM IODIDE SOLUTION. Dissolve 50 gm. of reagent grade sodiu iodide in 50 ml. of distilled water. Store in a brown glass bottle provide with a dropping pipette. Test the solution every week for the absence o free iodine by adding 1 ml. of it to 10 ml. of tungstate-phosphoric aci solution and then adding a drop of starch solution. If a blue color appears discard the solution and make up fresh.

1 PER CENT STARCH SOLUTION. Triturate 10 gm. of soluble starch wit cold water to a thin paste. Rinse into a liter of boiling water. Stir and boi for a minute. Cool. Add 50 ml. of a solution containing 6 gm. of zinc chlorid to prevent deterioration. Distribute the solution in tightly stoppered smal vials.

STANDARD 0.1000 *N* POTASSIUM IODATE SOLUTION. Weigh 3.567 gm of reagent grade potassium iodate. Transfer it quantitatively to a 1 lite volumetric flask. Make up to mark with water. Stopper and mix well. Transfe to a clean, dry bottle. This solution is stable.

STOCK STANDARD 0.9232 *N* SODIUM THIOSULFATE SOLUTION. Weigh 57.300 gm. of reagent grade, crystalline sodium thiosulfate ($Na_2S_2O_3.5H_2O$). Transfer it quantitatively to a 250 ml. volumetric flask. Make up to mark with water. Stopper and mix well. Transfer to a clean, dry bottle. The solution keeps indefinitely.

STANDARD 0.02308 *N* SODIUM THIOSULFATE SOLUTION. Pipette 25.00 ml. of the 0.9232 *N* sodium thiosulfate solution into a 1 liter volumetric flask. Add 1 gm. of borax as a preservative. Dilute to mark with water. Stopper and mix well. Transfer to a clean, dry, amber bottle. Check the accuracy of the solution after it is made up, and every week thereafter as follows: Pipette 5.00 ml. of the standard 0.1000 *N* potassium iodate solution into a 125 ml. Erlenmeyer flask. Add 20 ml. of water and 1.5 ml. of 1 *N* sulfuric acid. Add 1.5 ml. of the sodium iodide solution. Titrate (see the directions above) with the 0.02308 *N* sodium thiosulfate solution. The volume of thiosulfate required should be 21.67 ml. If it differs by more than 0.1 ml. from this value, add either water or 0.9232 *N* sodium thiosulfate in the calculated amount and titrate again.

(*a*) What is the "chloride shift"?

(*b*) If carbon dioxide is lost from the blood sample, will the chloride concentration of the serum become higher or lower?

(*c*) What is the clinical significance of serum chlorides?

(*d*) What is the effect of sunlight on sodium iodide solid or solution?

(*e*) Can a protein-free, Folin-Wu filtrate be used for the chloride determination? How?

(*f*) What other substance may be used to precipitate the serum proteins without interference in this determination?

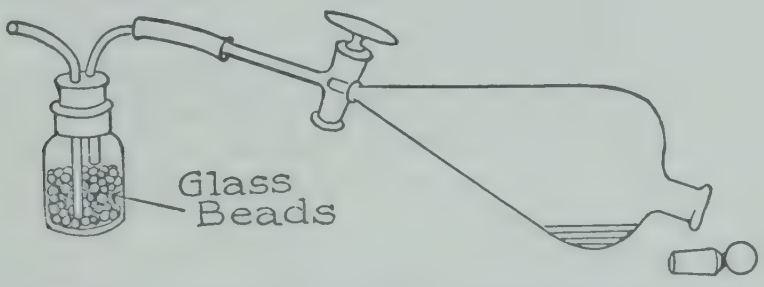

Fig. 21. Saturation of plasma with carbon dioxide.

Experiment 17. Carbon Dioxide Capacity.* The plasma from oxalated blood is rotated in a separatory funnel filled with air that has been exhaled from the lungs and whose carbon dioxide tension approximates that of normal arterial blood. By this treatment the plasma combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of this saturated plasma is introduced into the Van Slyke volumetric apparatus and acidified. The carbon dioxide is liberated quantitatively from

* Van Slyke and Cullen, *J. Biol. Chem.*, *30*, 289 (1917); Van Slyke, *J. Biol. Chem.*, *30*, 347 (1917); Van Slyke and Stadie, *J. Biol. Chem.*, *49*, 1 (1921); Van Slyke and Neill, *J. Biol. Chem.*, *61*, 297 (1924); Van Slyke, *J. Biol. Chem.*, *73*, 121 (1927).

the plasma in a vacuum. The volume of this gas is measured at atmosphe pressure, reabsorption being minimized by the removal of the aque liquid. A table provides corrections, and reduction of the gas volume standard conditions.

PROCEDURE. Centrifuge enough citrated or oxalated blood to provi the required amount of plasma. Transfer the cell-free plasma to a d separatory funnel (about 250 ml.) which is connected to the bottle of bea as indicated in Figure 21.

Without inspiring more deeply than normally, expire as quickly and co pletely as possible through the glass beads and separatory funnel, inserti the stopper of the funnel just before the expiration is finished, so that the is no opportunity for air to be drawn back into the funnel.

Close the stopcock. Remove the funnel from the bottle of glass beads a holding it in a horizontal position, rotate the funnel for three minutes, d tributing the plasma in a thin layer as completely as possible over t interior surface of the funnel. (During this procedure the plasma co bines with as much carbon dioxide as it is able to hold under normal carb dioxide tensions. The carbon dioxide tension of alveolar air approximat that of arterial blood.)

After saturation is complete, place the separatory funnel in an uprig position and allow the plasma to drain from the walls and collect in t contracted space at the bottom of the funnel.

(*a*) *Filling the apparatus.* Pour mercury into the levelling bulb until it three quarters full. (See Figure 22.) With both stopcocks open, fill t apparatus with mercury by raising the levelling bulb. Expel the air an some mercury into the cup, and by turning the upper stopcock, expel som mercury through the side arm. Close the upper stopcock.

(*b*) *Expelling the air.* With the upper stopcock closed, lower the levellin bulb so that the mercury column falls down to the level of the rubber tu ing attached to the apparatus. Tap the rubber tubing gently to release an air bubbles in the tubing. Raise the bulb to bring the mercury up again Rotate the lower stopcock so as to fill both the left and right glass tube with mercury. Expel any air together with some mercury through the sid arm.

(*c*) *Testing for a vacuum.* With both stopcocks closed, lower the levellin bulb so that the mercury drops below the bottom stopcock. Gently raise th bulb so that the mercury hits the stopcock. A metallic click will be heard i air is absent. (A dull sound indicates an air pocket and the directions in (*b*) must be repeated or else the stopcocks need to be regreased.) Open th lower stopcock, lower the bulb and gently bring it up again. A metalli click against the upper stopcock indicates an air-tight apparatus.

(*d*) *Analysis.*

1. Transfer 1 ml. of water to the cup.

2. Dip the pipette containing 1.00 ml. of the plasma below the water an allow the plasma to flow from the pipette to form a layer below the wate in the cup. Add 3 drops of caprylic alcohol.

3. Open the lower stopcock, lower the levelling bulb and carefully ope the upper stopcock so as to admit the plasma and most of the water into th

)paratus. Be careful not to admit any air into the bore of the stopcock. lose the stopcock.

4. Add 1 ml. of lactic acid to the cup. With the levelling bulb lowered, ırefully open the upper stopcock so as to admit most of the lactic acid, oiding air bubbles in the capillary. Close the stopcock.

5. Add some mercury to the cup and admit some into the apparatus, again oiding air bubbles. This will produce a mercury seal in the stopcock.

6. Lower the levelling bulb until the mercury column drops to the 50 ml. ark of the apparatus. Close the lower stopcock. Rest the levelling bulb. Re- ove the apparatus from the stand and grasp the two stopcocks securely

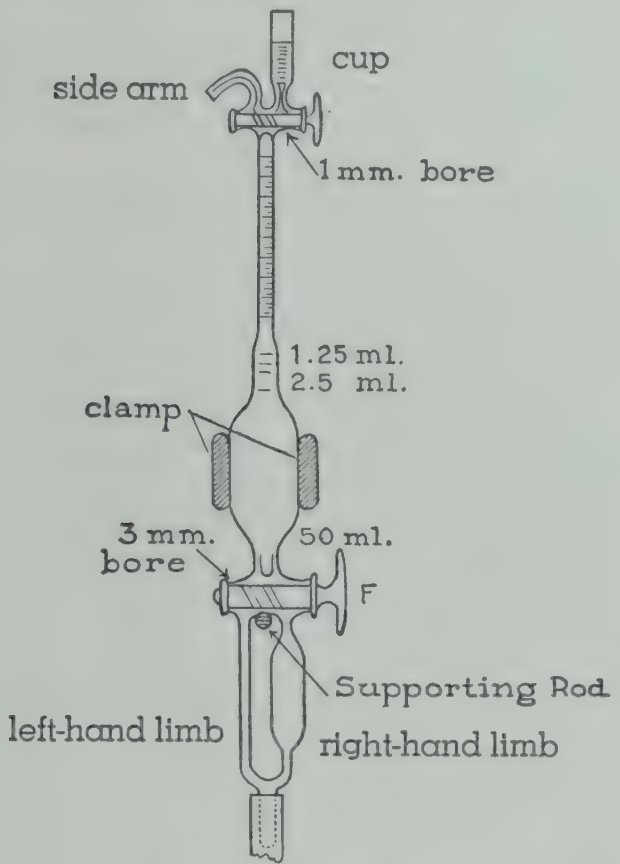

Fig. 22. Van Slyke pipette.

ith the thumb and forefingers of each hand. Holding the stopcocks securely o that they cannot be pushed loose, invert the apparatus about 15 times. Re- lace on the stand.

7. Lower the levelling bulb very far down and manipulate the lower stop- ock so that the small amount of mercury and most of the solution is drawn ıto the right-hand column below this stopcock. Now manipulate the lower topcock so that clean mercury from the left-hand column rises into the main hamber of the apparatus.

8. Adjust the mercury height of the levelling bulb so that it is at the same vel as the mercury height in the apparatus. Close the lower stopcock and ead the volume of carbon dioxide. Also read the temperature and the ıarometric pressure.

(*e*) *Cleaning the apparatus.* Lower the levelling bulb and allow the

Table 4

TABLE FOR CALCULATION OF CARBON DIOXIDE-COMBINING POWER OF PLASMA

Observed Vol. Gas × *B*/760*	*Ml. of CO_2 Reduced to 0°, 760 mm. Bound as Bicarbonate by 100 ml. of Plasma*				*Observed Vol. Gas* × *B/760*	*Ml. of CO_2 Reduced to 760 mm. Bound as Bicarbonate by 100 ml. of Plasma*			
(*A*)	15°	20°	25°	30°	(*A*)	15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.1
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.5	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.8	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

* B = barometric reading.

ıercury to run only into the left-hand tube below the lower stopcock. Now djust the stopcock and levelling bulb so that the solution in the right-hand imb flows up into the apparatus. Expel the solution and some of the mercury hrough the side arm. The apparatus is ready for the next analysis after ashing the cup with some water.

CALCULATION: $\frac{Volume\ of\ CO_2 \times Barometric\ Pressure}{760} = A$

Look up the volume of *A* in Table 4. That value, times 1.017, gives the olumes per cent of carbon dioxide.

REAGENTS:

MERCURY, redistilled.

CAPRYLIC ALCOHOL, C.P.

5 PER CENT LACTIC ACID. Dilute 5 ml. of concentrated C.P. lactic acid o 100 ml.

(*a*) What are some of the methods of studying the acid-base balance of he body?

(*b*) What are the objections to using whole blood instead of serum?

(*c*) Why is a metallic click obtained when the mercury is raised in a acuum against the stopcock?

(*d*) Why is the serum placed under water in the cup?

(*e*) Why is the solution removed before measuring the gas volume?

(*f*) When the mercury is wet, bubbles of gas often form and rise through he mercury column. Explain.

(*g*) Why is lactic acid used instead of a mineral acid?

(*h*) What other methods may be used for determining carbon dioxide?

(*i*) For what other determinations can this apparatus be used?

Experiment 18. Determination of the Oxygen Capacity of Blood.*

(*a*) Stir the special oxygen reagent to emulsify the caprylic alcohol. See previous experiment and Figure 22 for diagram of the apparatus and instructions as to its use. Introduce 7.5 ml. of the special reagent into the chamber of the apparatus, evacuate and shake. Remove air through the side arm of the upper stopcock.

(*b*) Transfer the blood to a separatory funnel and rotate to spread the blood in a thin layer so as to completely aerate it.

(*c*) Turn the upper stopcock and force 6 ml. of the gas-free reagent into the cup. By means of a differential pipette, add 2 ml. of the blood below the layer of the reagent and then allow it to flow into the chamber with all out 1 ml. of the reagent.

(*d*) Remove excess reagent from the cup with a medicine dropper and make a mercury seal. Evacuate the chamber and shake for 5-10 minutes.

(*e*) Create a slight negative pressure. Wash the cup with water and then ntroduce 0.5 ml. of 2 per cent NaOH (previously de-aerated). Admit this nto the chamber followed by a thin stream of mercury. Wait for 1 minute.

* From Hawk, Oser and Summerson, Practical Physiological Chemistry, The Blakiston Co., 1947.

(*f*) Bring the gas to barometric pressure. Read the gas volume, temp ture and barometric pressure.

(*g*) CALCULATIONS:

V = observed volume of gas ($O_2 + N_2$)
t = temp. in °C.
B = barometric pressure in mm. Hg.
W = aqueous tension (handbook).

$$\text{Volume \% } O_2 \text{ Capacity} = \left(\frac{(B - W)}{760\ (1 + 0.00367t)} \times \frac{100V}{2}\right) - 2.1$$

The value 2.1 corrects for O_2 and N_2 physically dissolved at atmosph pressure.

Hemoglobin (g. per 100 ml.) = 0.736 × vol. % O_2 capacity.

Special Oxygen Reagent:

Potassium ferricyanide	3.0 gm.
Saponin	3.0 gm.
Caprylic alcohol	3.0 ml.
Water to	1 liter.

Experiment 19. The Manometric Determination of Urea.* Slyke and his collaborators have perfected another piece of apparatus determining gas volumes. It is a manometric device in which the volume the gas is held constant while its pressure is read from a manometer. A high degree of accuracy can be obtained with this apparatus than with the vo metric one described in Experiment 17. The errors in the readings are lesser magnitude, smaller quantities may be used, calculations are simpl and finally it is easier to use. Many different determinations may be p formed in the apparatus. Methods have been worked out to determine blo or urine carbon dioxide, bicarbonate, oxygen-combining capacity, carb monoxide, urea, amino nitrogen, glucose, potassium, calcium, lactic ac lipoids and others. As an example of the use of the apparatus, the meth for determining blood urea will be described.

TO EMPTY, CLEAN AND FILL THE APPARATUS. Lower the levelling bu *C* (Fig. 23) to the floor and place it in a vessel to collect the mercury whi issues from it.

Open stopcock *c*, then *b*, then *a*, then *d*, and allow as much mercury possible to flow out of the apparatus.

Remove rubber tubing *D* and replace it with another rubber tube attach on the other end to a suction flask.

Apply strong suction. Draw water through the manometer *B* and t extraction chamber *A*, closing the appropriate stopcocks when necessary.

Remove all four stopcocks from their barrels and remove the grease.

Replace the ungreased stopcocks in their barrels. Slowly draw alcoho potassium hydroxide through all parts of the apparatus. **(Beware! Do n get alcoholic potassium hydroxide on the body or clothing.)**

After degreasing, draw acetone through the apparatus to wash out t

* Peters and Van Slyke, Quantitative Clinical Chemistry, Vol. 2, Williams and Wilki 1932.

alcoholic solution completely; then draw through a current of air until no trace of an odor of acetone remains.

Grease all stopcocks.

Disconnect the suction tube. Replace it with the rubber tube *D* and bevelling bulb *C*.

Close *d*. Pour clean mercury into *C*.

Raise *C* until the mercury passes through *a*. Close *a*.

When the mercury passes through *b*, close it.

Open *d*, allow mercury to pass through it, and then close it.

In order to remove any bubbles or film of air, lower *C* below Position 1 and evacuate the manometer, the chamber and tube *E*.

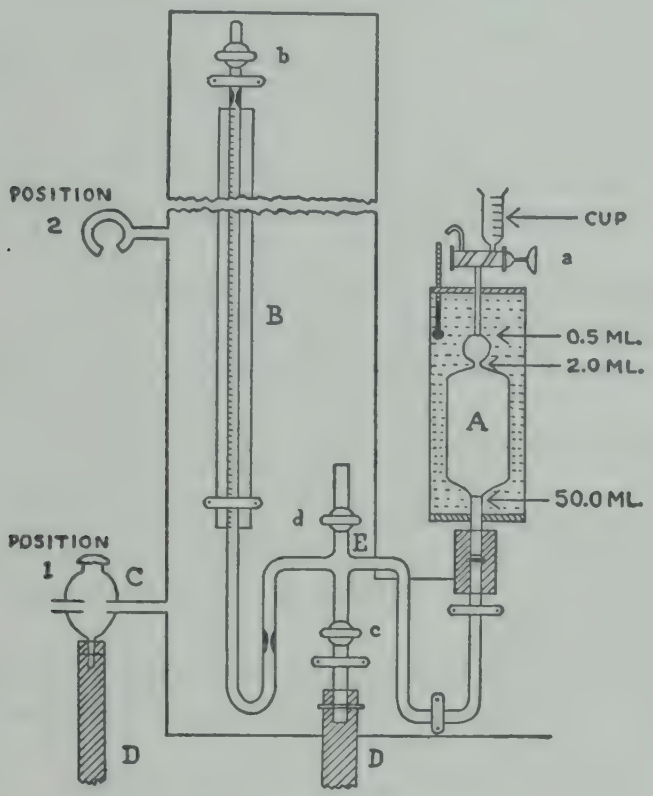

Fig. 23.

Raise *C* so that the apparatus fills with mercury. Expel air bubbles through the appropriate stopcocks. (*Note:* A gentle tapping of the glass during this process facilitates it.)

Repeat the de-aerating process until no more bubbles appear.

Place 1 ml. of ethylene glycol in the tube above *b*.

With *C* in Position 1, open *b* so slightly that the glycol slowly flows into the manometer to about 10 cm. mark. Then raise *C* to Position 2 until the mercury passes through *b*, which is then closed. Just sufficient dehydrating fluid should be left in the manometer to wet the wall, but not enough to obscure the mercury meniscus.

Warning. Never lower *C* below Position 1 while *a* is open. If this is done, the apparatus will have to be emptied, cleaned and refilled.

To Grease Stopcocks. Place *C* in Position 1. Open *a*. Remove, lubricate, and replace *a*.

Hang *C* a little below the level of stopcock *c*. Open *d*. Open *c*. Remo lubricate and replace *c*.

Note: Stopcocks *a* and *c* must always be well greased. They must t smoothly and hold a vacuum. A single lubrication should serve for a do analyses.

MANOMETRIC BLOOD UREA DETERMINATION. When the apparatus is in use, the extraction chamber *A* and the cup above it are kept filled w water. Before a determination the extraction chamber is cleaned. To do t raise *C* to Position 2. Open *c*. Barely opening *a*, allow the aqueous liquid the extraction chamber and a small amount of mercury to flow into the c Remove it with suction. Close *a*. Lower *C* below Position 1 until the mercu falls to the 50.0 ml. mark in chamber *A*. Close *c*. With *C* in Position 1, a intermittently opening and closing *a*, admit into the chamber 10 to 15 ml. water containing about 1 ml. of *N* lactic acid. Allow no air to enter. Shake chamber for 15 to 30 seconds. Raise the levelling bulb to Position 2 a eject the liquid as described above.

Now, with *A*, as well as the capillary tube above it, filled with mercu and all stopcocks closed, place 2 drops of caprylic alcohol in the cup of t apparatus.

Place *C* in Position 1. Open *c*. Cautiously manipulate *a* so that the capry alcohol fills the capillary below the cup.

Place 1 ml. of 0.1 *N* lactic acid in the cup and add water to the 4.5 m mark.

Fill a rubber-tipped, 1 ml. stopcock pipette, calibrated between mar with blood.

With the tip of the pipette snugly in the bottom of the cup beneath t liquid, open the stopcock of the pipette. Slowly open *a* until 1.00 ml. blood flows out of the pipette and into *A*. Close *a*. Close the stopcock of t pipette. Remove the pipette.

Open *a* and allow the acidified water in the cup to flow into *A*. Close

Lower *C* below Position 1 until the mercury in *A* reaches the 50 ml. mar Close *c*.

Place *C* in Position 1 and shake *A* for thirty seconds.

Place *C* in Position 2. Open *c*. When *A* is one-quarter filled with mercur open *a* without stopping the inflow of mercury.

When all gas has been driven out of the chamber and the liquid *just* fi the capillary above *a*, close *a*.

Repeat this extraction of gases from the blood and water twice agai

Place 1 ml. of 10 per cent urease suspension in the cup.

Discarding the first 3 drops, run into the urease suspension 0.25 ml. drops) of 0.26 *M* sodium phosphate from the soda-lime tube (Fig. 24 Place *C* in Position 1 (Fig. 23).

Immediately open *c*, then *a*, and draw the buffered urease suspension in *A*. (*Note:* No air may be admitted into *A* at this point in the procedure hereafter.) Close *a*.

Place a few drops of mercury in the cup. Manipulate *a* so that the mercu fills the capillary and stopcock bore.

Lower *C* below Position 1 until the mercury in *A* falls to the 50 ml. mark. Then raise *C* to Position 1, allowing the chamber to fill again.

Allow two minutes to pass so that the enzymatic action may be complete.

Place 0.5 ml. of *N* lactic acid in the cup. Manipulate *a* so as to draw 0.25 ml. of the acid into *A*. Remove excess acid with suction. Seal stopcock *a* with mercury.

Table 5

UREA IN BLOOD OR PLASMA BY MANOMETRIC UREASE METHOD

Temperature °C.	*Factor to Give Milligrams of Urea N per 100 Ml. of Blood* *Sample = 1 Ml.* *Gas Volume at Which Pressure Is Read*	
	0.5 Ml.	2 Ml.
15	0.0955	0.3740
16	47	12
17	40	0.3684
18	33	59
19	26	34
20	19	08
21	13	0.3583
22	08	58
23	02	34
24	0.0895	10
25	89	0.3488
26	83	66
27	77	45
28	72	24
29	68	04
30	63	0.3384
31	58	66
32	54	48
33	49	29
34	44	11

Lower *C* below Position 1. When the mercury in *A* reaches the 50.0 ml. mark, close *c*.

With *C* in Position 1, shake *A* for two minutes.

Open *c* with a smooth, gradual motion so that the mercury in chamber *A* rises at a fairly rapid rate through the middle three-fourths of the chamber. Then gradually close *c* to reduce the rate of flow of the mercury, making it so slow that by the time the 0.5 ml. mark is reached there is no difficulty in stopping exactly at the mark. (*Note:* Avoid all jerky starting and stopping of the mercury flow. The entire operation should take about forty seconds. If the aqueous meniscus is not stopped exactly at the mark, readjustment should not be attempted. Instead, lower the mercury again to the 50 ml. mark, shake

the chamber for one minute, and then exactly adjust the gas volume to ml. If the urea content is over 75 mg. per cent [35 mg. per cent urea nitrogen], the carbon dioxide pressure at the 0.5 ml. volume will exceed 400 m In this case, lower the mercury in *A* to the 50.0 ml. mark, shake for minute, and adjust the gas volume to 2.0 ml.)

Read the pressure on the manometer *B*. This reading is P_1.

Place 4 drops of 5 *N* sodium hydroxide in the cup. Manipulate stopcoc so that the alkali enters chamber *A* slowly. (Admit no air.)

Open *c* so as to wash the alkali out of the narrow tube below *a*.

Lower *C* below Position 1 and allow the surface of the aqueous solut to fall below the 0.5 ml. or 2.0 ml. mark used for the P_1 reading.

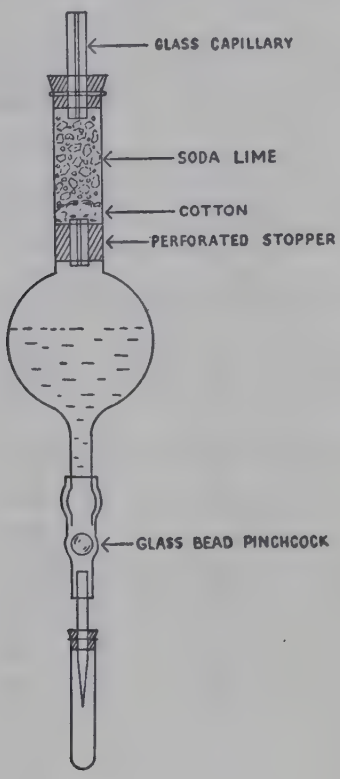

Fig. 24.

Close *c*. Place *C* in Position 1. Open *c* so that the gas volume is brou to that used in reading P_1. Close *c*.

Read the pressure on the manometer. This reading is P_2.

Read the temperature on the thermometer in the water jacket of *A*.

Wash out the chamber as directed above.

Perform a blank analysis exactly as directed for blood, replacing the blo with water. The difference between P_1 and P_2 in the blank analysis is

CALCULATIONS. $P_{urea} = P_1 - P_2 - c$.

Look up the value of P_{urea} at the appropriate temperature in Table 5 whi will give the factor by which P_{urea} is multiplied to give the milligram p cent of urea nitrogen.

REAGENTS:

18 *N* SODIUM HYDROXIDE (SATURATED, CARBON DIOXIDE-FREE). D solve 500 gm. of sodium hydroxide in 500 ml. of water, cooling if necessa

Permit the solution to stand in a paraffined bottle until the carbonate has settled.

5 *N* Sodium Hydroxide. Dilute 27 ml. of the 18 *N* sodium hydroxide to 100 ml. Keep protected from atmospheric carbon dioxide by immediately drawing the solution into a soda-lime tube such as shown in Figure 24.

N Lactic Acid. Dilute 50 ml. of lactic acid—specific gravity, 1.20—to 500 ml. Bubble purified air through this solution for fifteen minutes. Transfer to a clean, dry, glass-stoppered bottle.

0.1 *N* Lactic Acid. Dilute 50 ml. of *N* lactic acid to 500 ml. Aerate for fifteen minutes and then transfer to a clean, dry, glass-stoppered bottle.

0.26 *M* Sodium Phosphate Solution. In a 100 ml. volumetric flask place 1.75 ml. of syrupy phosphoric acid, specific gravity 1.72 to 1.73. Fill the flask half-full with water. Add two drops of 1 per cent phenolphthalein solution. From 10 ml. burette, add 18 *N* carbonate-free sodium hydroxide until the solution turns pink. Note the volume of sodium hydroxide added. Add exactly half as much more. (The total volume should be about 4.3 ml.) Immediately after the addition of alkali, dilute the solution to mark, stopper the flask, mix the solution, and then draw it up into a soda-lime tube such as shown in Figure 24.

10 Per Cent Urease Solution in 50 Per Cent Glycerol. Dissolve 1 gm. of Squibb's Urease preparation in 5 ml. of water. Add 5 ml. of glycerol. Mix. The enzyme solution should be prepared the same day it is to be used.

Experiment 20. The Determination of Hemoglobin. Three general methods are used for the estimation of hemoglobin in blood. The most accurate, but by far the most elaborate, is the gasometric method whereby the oxygen or carbon monoxide-combining capacity of the blood sample is measured in the Van Slyke manometric apparatus (see p. 116). From such data, and a knowledge of the quantity of oxygen or carbon monoxide capable of combining with 1 gm. of hemoglobin, the concentration of the hemoglobin in the blood sample may be calculated. However, the technic is too difficult and the method too time-consuming to be used for routine purposes.

The second group of methods depends on the color of oxyhemoglobin in the blood, or the color of one of its derivatives. These are the methods most generally used for routine analysis. Some accuracy is sacrificed here for the sake of simplicity and speed.

The easiest of these methods is that of Tallqvist, in which a drop of blood is absorbed on paper and then its color is compared with that of colored paper standards. The error of this method may be as high as 20 per cent.

In the Dare Hemoglobinometer, a drop of blood is spread to uniform thickness on a specially designed slide. Its color is compared with a glass standard.

The Sahli method has as its principle the comparison of the color of acid hematin formed from a given sample of blood with a standard acid hematin solution. A small quantity of the blood is mixed with 0.1 *N* hydrochloric acid in a graduated tube. After a minute the sample is diluted until its color matches a standard. Since acid hematin color fades, a glass standard is usually substituted for the solution.

Newcomer modified the above method so that comparisons could made in the Duboscq colorimeter. The glass standard, placed under one c was carefully chosen after a spectroscopic analysis.

In other methods the blood is quantitatively diluted with a weakly alkal solution. The color of the oxyhemoglobin is then compared with that o standardized blood sample.

Such standardized samples soon change in properties. Since it is possible to obtain pure hemoglobin from which standards may be made, all the methods of this group the "standards," artificial or natural, must checked with blood samples analyzed by the Van Slyke oxygen or carb monoxide-combining capacity method.

A source of confusion in the aforementioned methods is that the resu are usually given in terms of per cent, not of hemoglobin, but of "norma hemoglobin. Various authorities and manufacturers differ in their choice the normal value. This is possible since normals range from 14 to 16.5 g of hemoglobin per 100 ml. of blood for men and from 13 to 16 for wome It would be far better to give the results in terms of grams of hemoglobin p 100 ml. of blood. A table of standards is given here. From it one can see th a sample of blood containing 13 gm. of hemoglobin per 100 ml. would designated as 94 per cent by the Dare method and only 75 per cent by t Sahli method.

Standards in use:	Dare	100 per cent = 13.77 gm.
	Haldane	100 per cent = 13.80 gm.
	Newcomer (Williamson)	100 per cent = 16.92 gm.
	Oliver	100 per cent = 15.00 gm.
	Sahli	100 per cent = 17.20 gm.
	Tallqvist	100 per cent = 15.80 gm.
	Von Fleischl-Nueschen	100 per cent = 15.80 gm.

Finally, advantage has been taken of the fact that hemoglobin contai iron to the extent of 0.335 per cent, to design quantitative methods for i determination. The accuracy of these methods lies between those of the fi two groups.

Wong Method.* The iron of the hemoglobin molecule is detach by the action of concentrated sulfuric acid, decomposition being aided the use of potassium persulfate. The proteins are then precipitated by tun stic acid and filtered. Color is produced by the action of potassiu thiocyanate and compared with a standard iron solution treated in the sam way.

PROCEDURE. Pipette 0.50 ml of well-mixed whole blood into a 50 m volumetric flask and add 2 ml. of iron-free concentrated sulfuric acid.

Whirl the flask, to agitate the mixture, for one minute.

Add 2 ml. of a saturated solution of potassium persulfate and mix.

Dilute to about 25 ml. with distilled water and add 2 ml. of 10 per ce sodium tungstate solution.

Cool to room temperature under the tap and dilute to volume with distille water.

* Wong, *J. Biol. Chem.*, 77, 409 (1928).

Stopper the flask and invert three or four times to effect thorough mixing. Filter through a dry filter paper into a dry 125 ml. Erlenmeyer flask.

Transfer 20.00 ml. of the clear filtrate to a large test tube graduated at 25 ml.

Measure into another similar tube 1.00 ml. of a standard solution containing 0.10 mg. of iron per milliliter. Add water until the total volume is about 18 to 20 ml.

Add 0.8 ml. of iron-free, concentrated sulfuric acid to each tube.

Cool to room temperature under the tap.

Now add to both the standard and the unknown 1 ml. of a saturated potassium persulfate solution and 3 ml. of a 3 *N* potassium thiocyanate solution.

Dilute to the 25 ml. graduation. Stopper. Mix and compare in a colorimeter.

Calculate the number of milligrams of iron per 100 ml. of blood. From this value calculate the number of grams of hemoglobin per 100 ml. of blood. If a visual colorimeter was used, refer to page 67 for the equation. If the determination was performed photocolorimetrically, the appropriate relationships may be found on page 71, or a calibration curve may be used.

When performing the determination photocolorimetrically, good results will be obtained with a green filter having a maximal transmission at 550 mμ.

REAGENTS:

CONCENTRATED SULFURIC ACID (must be iron-free).

SODIUM TUNGSTATE. 10 per cent solution (see p. 80).

SATURATED POTASSIUM PERSULFATE. Introduce into a 1 liter glass-stoppered bottle 70 gm. of pure potassium persulfate and shake with 1 liter of distilled water.

POTASSIUM THIOCYANATE. Prepare an approximate 3 *N* solution by dissolving 146 gm. of the pure salt with distilled water in a 500 ml. volumetric flask and making up to the mark. Filter if necessary.

STANDARD IRON SOLUTION. Weigh accurately 0.861 gm. of crystallized ferric ammonium sulfate and dissolve in about 50 ml. of distilled water. Add 20 ml. of 10 per cent sulfuric acid. Dilute with distilled water to 1 liter. Each milliliter will contain 0.10 mg. of iron.

(*a*) Why is potassium persulfate used in the determination of iron?

(*b*) What other methods can be used for the determination of hemoglobin in the blood?

Experiment 21. The Determination of Total Proteins in Serum.* Diluted serum is made alkaline with sodium hydroxide and then treated with Folin and Ciocalteu's phenol reagent. The blue color which develops is due to the reduction of the phosphomolybdates and phosphotungstates in the reagent by phenolic groups (tyrosine) in the proteins. Since the percentage of these groups in serum proteins is fairly constant, the color is proportional to the protein concentration. As a standard, tyrosine is used, protein solutions

* Greenberg, *J. Biol. Chem.*, *82*, 545 (1924).

not being sufficiently stable. The color given by 1 mg. of tyrosine is equivale to that of 16 mg. of total protein (a mixture of the serum albumins a globulins).

PROCEDURE. Pipette 1.00 ml. of fresh serum and 9.00 ml. of 0.9 p cent sodium chloride solution into a dry test tube.

Stopper and mix well by slowly inverting the tube several times.

Transfer 2.00 ml. of the diluted serum to a 50 ml. volumetric flask.

Into a second, 50 ml. volumetric flask, pipette 4.00 ml. of standard tyr sine solution.

****Add about 25 ml. of water to each flask.

Add 2 ml. of 5 *N* sodium hydroxide to each flask and mix well.

Add, slowly and with constant agitation of the flask, 3 ml. of phen reagent from a Mohr pipette.

Dilute the contents of each flask with water to the 50 ml. mark.

Stopper both flasks and mix thoroughly by inverting many times.

Allow the color to develop for five minutes.

Read in the colorimeter within the next thirty minutes.

When performing the determination photocolorimetrically, good resul will be obtained with a red filter having a maximal transmission at 640 mμ. or, if the instrument is not sufficiently sensitive to give adequate readings a this wavelength, a green or even a blue filter may be used.

Calculate the number of grams of protein per 100 ml. of serum. If a visua colorimeter was used, refer to page 67 for the equation. If the determinatio is performed photocolorimetrically, the appropriate relationships may b found on page 71, or a calibration curve may be used.*

REAGENTS:

0.9 PER CENT SODIUM CHLORIDE. Dissolve 9 gm. of sodium chloride i a liter of water.

5 *N* SODIUM HYDROXIDE. Dissolve 200 gm. of sodium hydroxide in liter of water.

PHENOL REAGENT (FOLIN AND CIOCALTEU). Place in a 2 liter Florenc flask, 100 gm. of reagent grade sodium tungstate and 25 gm. of reagent grad

* Kingsley devised a method for determining serum proteins by means of the biure reaction. The advantage is that the reaction does not depend on the presence of a par ticular amino acid (e.g., tyrosine) in the protein but only upon its peptide linkages Whereas the percentage of an amino acid in a protein may vary, the percentage o peptide linkages is fairly constant.

The Kingsley Biuret method has been varied and modified by others. References are Kingsley: *J. Lab. Clin. Med., 27,* 840 (1942); Robinson and Hogden: *J. Biol. Chem., 135* 707, 727 (1940); Mehl: *J. Biol. Chem., 157,* 173 (1945); Summerson: Hawk, Oser an Summerson, Practical Physiological Chemistry, The Blakiston Co., 1948.

Essentially, serum is diluted with saline and then treated with a special biuret reagen to produce a color the intensity of which is proportional to the protein content. Serum albumins may be separated from the serum by precipitating the serum globulins wit 23 per cent sodium sulfate solution as in the above method. Separation is facilitated b adding ether, shaking and then centrifuging. The manipulations are so similar to thos of the Greenberg method that further details are being omitted.

The standard used is pooled serum, the protein content of which is determined by th Kjeldahl method; or bovine albumin, which is obtainable from The Armour Co., Chicago Illinois.

dium molybdate. Add 700 ml. of water and 50 ml. of 85 per cent syrupy osphoric acid. Mix and add 100 ml. of concentrated hydrochloric acid. ix again. Reflux gently for ten hours. Now add 150 gm. of lithium sulfate. ix until the solid dissolves. Add 5 to 10 ml. of bromide water. Mix. Boil r fifteen minutes to remove the excess bromine. Cool to room temperature. ransfer to a liter volumetric flask. Dilute to mark with water. Mix thor- ghly. Filter, if not perfectly clear, into a glass-stoppered amber bottle. The agent is sensitive to light and dust. It should have a dirty yellowish color. it becomes green, it should be discarded.

22.2 PER CENT SODIUM SULFATE SOLUTION. Transfer 222.0 gm. of pure nhydrous sodium sulfate to a liter volumetric flask. Add about 700 ml. of istilled water that has been warmed to 50° to 60°C. Rotate the flask until l the salt dissolves. If necessary, cool to 40°C. and add water to the mark. topper the flask. Mix thoroughly. Transfer to a bottle. Store at 37°C., since ie solute will crystallize out at lower temperatures. If this happens, warm ie bottle and shake until the solid dissolves.

STANDARD TYROSINE SOLUTION. Weigh 0.2000 gm. of pure tyrosine. ransfer it to a liter volumetric flask, using 0.1 *N* hydrochloric acid to aid n the transfer. Dilute to mark with the 0.1 *N* hydrochloric acid. Stopper. Mix thoroughly. Store in an amber bottle. This solution contains 0.20 mg. f tyrosine per milliliter. One milliliter of it will give the same color when reated with phenol reagent as 3.20 mg. of serum total proteins, or 3.36 mg. f serum albumins.

Experiment 22. The Determination of Serum Albumins and Globulins.* Sufficient 22.2 per cent sodium sulfate solution is added to diluted erum to precipitate the globulins. At this salt concentration the serum lbumins remain in solution. After removing the globulins, the protein in the iltrate is determined by means of Folin-Ciocalteu's phenol reagent (see bove). Tyrosine is used as a standard; 1.0 mg. gives a color equivalent to 6.8 mg. of albumin.

The serum globulin concentration is calculated as the difference between he serum total protein and the serum albumin concentration.

The amount of sodium sulfate added to the diluted serum and the tempera- ure of precipitation are critical. If the salt concentration is raised, more rotein precipitates and is called "globulin"; the "albumin" determination n this case will be low. The reverse condition also will occur. Since the atio of serum albumin to serum globulin is often of diagnostic significance, slight changes in the prescribed conditions will yield greatly altered albumin-globulin ratios.

PROCEDURE. Pipette 0.50 ml. of fresh serum into a dry test tube.

Add 9.50 ml. of 22.2 per cent sodium sulfate solution. (Since sodium sulfate crystallizes from this solution at room temperature, it should be stored in an incubator at 37°C. Also, the pipette should be warmed slightly o prevent crystallization in it.)

Stopper the tube and mix its contents by several slow inversions.

Incubate at 37°C. for at least four hours.

* Greenberg, *J. Biol. Chem.*, *82*, 545 (1924).

Set up in the incubator a dry funnel containing a dry, folded filter pap and beneath it place a dry 125 ml. Erlenmeyer flask.

Filter and refilter the mixture until a clear, colorless filtrate is obtain (Be careful to prevent excess evaporation at the elevated temperature covering the funnel with a watch glass.)

Pipette 5.00 ml. of the filtrate into a 50 ml. volumetric flask.

Into a second 50 ml. volumetric flask, pipette 4.00 ml. of standard ty sine solution.

Continue the procedure exactly as directed under The Determination Serum Proteins (Experiment 20, p. 124), from the asterisks to the end.

REAGENTS. See pp. 124, 125.

Experiment 23. The Determination of the Icterus Index.* A rap and approximate estimation of bilirubin in serum is made by the simp comparison of the color of the serum with that of a dilute standard potassiu dichromate solution. The presence of other serum pigments will make t icterus index appear too high, as will hemolysis of the blood sample.

PROCEDURE. Obtain a blood sample taken during the postabsorpti state so as to minimize interference by lipochromes. (Carotene-containi foods should have been avoided for the twenty-four hours before the dete mination.)

Allow the blood to clot.

Loosen the clot with an applicator, centrifuge the sample, and then dra off the serum. (Take every precaution to prevent hemolysis.)

Place about 5 ml. of 0.01 per cent potassium dichromate solution in dry test tube.

Pipette 1.00 ml. of the serum into a second similar dry test tube.

Dilute the serum with successive 1.00 ml. additions of 0.9 per ce sodium chloride solution, mixing after each addition, until the color appro imately matches the standard. Note the dilution.

Transfer the samples to dry colorimeter cups and compare in a colorimete

Calculate the icterus index on the basis that 1 unit of icterus index is serum color equal to that of 0.01 per cent potassium dichromate solutio

It is inadvisable to perform this determination photocolorimetrically. Th effect of the opalescence of most serum samples, which is automaticall compensated for by the naked eye, will cause some dispersion of light an high results when measured in a photoelectric colorimeter.

Experiment 24. The Determination of Bilirubin (*van den Bergh Test*). Diluted serum is treated with diazotized sulfanilic acid (*Ehrlich's* reagent in the presence of methyl alcohol. The bilirubin couples with the diazoniu salt to form an azo dye of pink color, the intensity of which is proportiona to the concentration of the bilirubin. Some or all of the bilirubin in ser appears to be conjugated with protein. Such compounds give a delaye reaction with *Ehrlich's* reagent, and the developed color is at first yellowish

* Rhamy and Adams, *J. Lab. Clin. Med.*, *13*, 87 (1927–28).

† Malloy and Evelyn, *J. Biol. Chem.*, *119*, 481 (1937); Sepulveda and Osterberg *J. Lab. Clin. Med.*, *28*, 1359 (1943).

rown, changing, only after one to two hours, to the characteristic pink-red olor of azorubin. The addition of methyl or ethyl alcohol to the diluted erum apparently causes the conjugated bilirubin to be freed for immediate oupling with the diazo reagent. This determination, therefore, gives the esult in terms of total bilirubin, or the so-called "indirect reaction."

PROCEDURE. Pipette 1.00 ml. of unhemolyzed serum into a 10 ml. olumetric flask.

Dilute to mark with distilled water, stopper and mix thoroughly.

Pipette into each of two dry labeled test tubes, 5.00 ml. of absolute ethyl alcohol and 1.00 ml. of *freshly prepared* diazo reagent.

Immediately transfer to one test tube, 4.00 ml. of the diluted serum.

Into the second tube, pipette 4.00 ml. of dilute standard bilirubin solution o. 1.

Stopper the tubes and mix the contents by slowly inverting them several mes. (Gas may be evolved. Why?)

Allow to stand for thirty minutes.

Compare the solutions in a colorimeter immediately thereafter.

Since the range of concentration of bilirubin is large, it is advisable to ake up several standards of various concentrations and to compare the nknown with the standard which most closely matches its color.

Calculate the number of milligrams of bilirubin per 100 ml. of serum. When using the visual colorimeter, refer to the equation on page 67. If a hotoelectric colorimeter is used, the appropriate relationships may be found n page 71, or a calibration curve may be used.

CALIBRATION CURVE. Place nine dry test tubes in a rack. Pipette the ollowing quantities of bilirubin standard solutions and absolute methyl lcohol into them:

	Mg. Per Cent of Bilirubin	*Ml. of Bilirubin Standard No. 1*	*Ml. of Bilirubin Standard No. 2*	*Ml. of Alcohol*
.....	0.00	0.00	..	9.00
.....	1.25	1.00	..	8.00
.....	2.50	2.00	..	7.00
.....	5.00	4.00	..	5.00
.....	7.50	6.00	..	3.00
.....	10.0	8.00	..	1.00
.....	15.0	..	3.00	6.00
.....	20.0	..	4.00	5.00
.....	25.0	..	5.00	4.00

Add 1.00 ml. of freshly prepared diazo reagent to each.

Stopper the tubes and mix the contents by slowly inverting several times.

Allow to stand for thirty minutes.

Read in the photoelectric colorimeter immediately thereafter, using the ppropriate filter. One with maximal transmission in the green at 535 mμ will ive good results.

Prepare a calibration curve on linear or semilog graph paper by plotting ie data.

REAGENTS:

METHYL ALCOHOL. Absolute, reagent grade.

Diazo Reagent. Solution *A*. Transfer 0.5 gm. of reagent gra sulfanilic acid to about 200 ml. of water. Add 7.5 ml. of concentrated hyd chloric acid. Stir until the solid is dissolved. Transfer to a 500 ml. vo metric flask. Dilute to mark with water. Mix. Store in an amber bottle.

Solution *B*. Dissolve 0.5 gm. of reagent grade sodium nitrite in 100 of water. This solution is stable for only one day.

Reagent. Must be prepared fresh just before use. Mix 25 ml. of so tion *A* with 0.75 ml. of solution *B*.

Stock Bilirubin Solution. Weigh 0.0100 gm. of pure biliru and transfer it to a dry 100 ml. volumetric flask, using reagent gra anhydrous chloroform to aid in the transfer. Dilute to mark with the chlo form. Stopper the flask. Mix thoroughly. Transfer to a dry, glass-stoppe amber bottle. This solution contains 0.10 mg. of bilirubin per milliliter.

Standard Bilirubin Solution No. 1. Transfer 5.00 ml. of sto bilirubin solution to a dry 100 ml. volumetric flask. Dilute to mark w absolute methyl alcohol. Stopper, mix thoroughly, and transfer to a d amber bottle. This solution contains 0.0050 mg. of bilirubin per millilit

Standard Bilirubin Solution No. 2. Transfer 20.00 ml. of sto bilirubin solution to a dry 100 ml. volumetric flask. Dilute to mark w absolute methyl alcohol. Stopper, mix thoroughly, and transfer to a d amber bottle. This solution contains 0.020 mg. of bilirubin per milliliter.

Chapter 13 **Urine**

URINE contains (along with a number of inorganic ions) nitrogenous compounds such as urea, creatinine and uric acid. Tests for these constituents are given, as well as several important quantitative determinations.

Experiment 1. Urea. Decomposition by Urease. To 3 ml. of 0.1 *M* urea add 4 drops of 0.04 per cent phenol red solution. If the pink alkaline color is not obtained, add 0.1 *M* sodium hydroxide, drop by drop, until the solution turns faintly pink. Add 0.1 *M* acetic acid, drop by drop, until the pink color is just discharged (*p*H 7). Add 0.5 gm. of soy bean (or Jack bean) meal, and shake. Allow to stand for some time. Note the color of the solution. Explain.

(*a*) Why must the *p*H be carefully adjusted?
(*b*) What effect has the presence of heavy metals?
(*c*) This reaction is the basis for a quantitative determination of urea. Explain.

Experiment 2. Urea. Urea Oxalate. Place 2 drops of 0.1 *M* urea on a microscope slide. Add 1 drop of 0.5 *M* oxalic acid and mix by stirring with a fine glass rod. Place a cover slip over the liquid and examine the crystals under a microscope. Make a drawing of the crystals.

(*a*) What is the formula of urea oxalate?
(*b*) How could you recover urea from urea oxalate?

Experiment 3. Urea. Formation of Biuret. Carefully heat 1 gm. of urea in a dry test tube. The urea melts. Note the odor of the gas which is evolved. Continue heating until the solidification point. Allow to cool. Add 2 ml. of water and shake well. Perform a biuret test on the solution.

(*a*) Write the equation for the reaction.
(*b*) What products other than biuret are formed?

Experiment 4. Xanthydrol Test for Urea in Urine.* Dilute 1 ml. of urine with water to 500 ml. Transfer 5 ml. of the diluted urine to a test tube, and add 5 ml. of glacial acetic acid and 0.5 ml. of a methyl alcohol solution of xanthydrol. Mix the contents thoroughly, preferably by closing the tube with a clean cork and shaking vigorously. Allow to stand for one hour, during which time the dixanthhydryl urea separates slowly from solution and gathers in large, loose clumps.

Experiment 5. Uric Acid. Murexide Test. Place 3 drops of concentrated nitric acid and 0.1 gm. of uric acid in a porcelain dish. Heat on a

* Allen and Luck, *J. Biol. Chem.*, *82*, 693 (1929).

steam bath until the nitric acid and water have evaporated. Note the color the deposit. Add 2 ml. of dilute ammonium hydroxide (1:100). Note t color.

Experiment 6. Uric Acid. Schiff Test. To 0.1 gm. of uric acid a 10 ml. of 0.1 *M* sodium carbonate. Pour solution on a filter paper moisten with 0.1 *M* silver nitrate. Note the color.

(*a*) What ions interfere with this test?
(*b*) Why cannot sodium hydroxide be used in place of sodium carbonat
(*c*) Does uric acid have reducing properties?

Experiment 7. Creatinine. Picric Acid Test. Place 5 ml. of urine one test tube and 5 ml. of water in another. To each add 1 ml. of a saturat solution of picric acid, and 1 ml. of 2 *M* sodium hydroxide. Note the colo

(*a*) Compare this test with that in which glucose is treated with alkali picrate solution.
(*b*) Are the colored compounds the same?

Experiment 8. Creatinine. Nitroprusside Test. To 5 ml. of urin add 5 drops of 0.1 *M* sodium nitroprusside. Add *M* sodium hydroxide, dro by drop, until a red color appears. Boil. Note the color change. Acidify car fully with glacial acetic acid and heat for one minute. Note the result.

(*a*) What substance in urine may interfere?
(*b*) What is the effect of alkali on creatinine?
(*c*) What products are formed when creatine is boiled with barium hydroxide?
(*d*) How may creatine be converted into creatinine?

Experiment 9. Phosphates. Add dilute ammonium hydroxide (1:10 to 10 ml. of urine until it is alkaline to litmus. Gently warm the mixture an filter (precipitate *A*). Add 3 ml. of "magnesia mixture" to the filtrate an warm gently. Note the precipitate (*B*).

(*a*) What are the precipitates *A* and *B*?
(*b*) What happens when urine is allowed to stand for some time?

Experiment 10. The Isolation of Hippuric Acid. The greater part o benzoic acid which is ingested is detoxified by conjugation with glycine, an the resulting hippuric acid is excreted in the urine. Hippuric acid is con veniently isolated by acidifying the urine and salting out with ammonium sulfate.

Ingest 1.4 gm. of sodium benzoate during the evening meal.

Collect all the urine voided in the morning. Dilute the urine in an appro priate-sized beaker with an equal volume of saturated solution of ammonium sulfate. Add 1.5 ml. of concentrated sulfuric acid for each 100 ml. of urine Stir the solution. Be sure that the solution is definitely acid to Congo red

Set the beaker aside—preferably in a refrigerator—for twenty-four hours or longer.

Filter the crystals on a small funnel, using suction. Wash the crystals with a small quantity of cold water, then air dry the residue, and finally dry it in an oven at 100°C. Weigh the crystals. Determine the melting point of the product. Recrystallize from the smallest amount of boiling water, and again determine the melting point of the crystals.

(*a*) Calculate the per cent "yield" of crude hippuric acid.

(*b*) Compare the melting point you obtained with that reported in the literature.

(*c*) What do you conclude about the purity of your hippuric acid?

(*d*) How would you proceed to purify it sufficiently to duplicate the melting point reported in the literature?

(*e*) In what other manner does the body detoxify benzoic acid?

(*f*) Compare your "yield" of hippuric acid with those of the other students.

TESTS FOR ABNORMAL CONSTITUENTS

The following tests may be performed on "pathological urine." The amounts of these constituents are too small to be detected in normal urine. For comparison, each of the tests may be done on normal urine.

Experiment 11. Test for Acetone Bodies (Rothera's Method). Saturate 10 ml. of urine with solid ammonium sulfate. Add 2 to 3 drops of a freshly prepared 5 per cent sodium nitroprusside solution and 1 to 2 ml. of concentrated ammonium hydroxide. Mix well and allow to stand for one-half hour.

A positive test is shown by the development of a purplish color approximating that of a permanganate solution. This test is given by acetone and acetoacetic acid.

Experiment 12. Gerhardt's Ferric Chloride Test for Acetoacetic Acid. To 5 ml. of urine, which contains no phenol, sodium bicarbonate, aspirin or other salicylates, add a 5 per cent ferric chloride solution, drop by drop, until no further precipitation of ferric phosphate occurs. Filter, and discard the precipitate. The development of a red color in the solution indicates the presence of acetoacetic acid. To confirm this, do the following: Boil another 10 ml. portion of the urine for about five minutes to decompose the acetoacetic acid. Cool. Repeat the test with 5 ml. of the cool, boiled urine. A negative result should now be obtained.

(*a*) Define the term "acetone bodies."

(*b*) What is the metabolic origin of acetone bodies?

(*c*) What is the range of the amounts of acetone bodies in normal urine?

(*d*) In what pathological condition is an abnormal amount of acetone bodies found in the urine? Why?

(*e*) How is the presence of excessive amounts of acetone bodies in th urine and blood related to acidosis?

Experiment 13. Test for Bile in Urine. See p. 62.

(*a*) What is the significance of the presence of abnormal amounts of bil pigments in the urine?

Experiment 14. Test for Blood in Urine. See p. 64.

Experiment 15. Test for Reducing Sugars in Urine. Add 5 ml. o Benedict's qualitative reagent to a test tube containing 8 drops of urine Place in a vigorously boiling water bath for five minutes, and allow to coo slowly.

Record the color of the precipitate, if any, and of the supernatant liquid

The phenylhydrazine reaction (p. 3) may be used to confirm the presenc of urinary glucose. Dilute 5 ml. of urine with 5 ml. of water and follow thos directions. Examine the crystals under the microscope.

(*a*) Is the presence of detectable amounts of glucose in urine a certain indication of diabetes? Why?

(*b*) What is meant by "the renal threshold"?

(*c*) What amounts of glucose may be found in 24 hour urine samples from patients with pathological glycosuria?

Experiment 16. Test for Proteins. For Heller's Ring Test, see p. 23.

Experiment 17. Coagulation Test for Proteins. Filter 10 ml. of urine, if it is not clear. Add 5 ml. of this clear urine to a test tube and bring to a boil. Keep at the boiling temperature for one to two minutes. When a precipitate forms, add 3 to 5 drops of 2 *N* acetic acid. A precipitate, which becomes more flocculent upon the addition of the dilute acetic acid, indicates the presence of proteins.

(*a*) What types of proteins are precipitated in the coagulation test?

(*b*) What happens when too much acetic acid is used in the coagulation test even though proteins are present?

(*c*) Occasionally a precipitate is obtained on heating the urine which dissolves upon the addition of several drops of 2 *N* acetic acid. What is this precipitate?

(*d*) Is the presence of albumin in the urine always an indication of a pathological condition?

Experiment 18. Test for Indican. To a test tube containing 10 ml. of urine, add 10 ml. of Obermayer's reagent and 3 ml. of chloroform. Mix by inverting the test tube about ten times. Allow to stand and examine the chloroform layer.

(*a*) Describe the chemistry of this test.

(*b*) State a condition that causes indicanuria.

QUANTITATIVE DETERMINATIONS

Experiment 19. Collection of a 24 Hour Urine Specimen. The student will obtain some toluene or a few crystals of thymol (see instructor). The preservative is to be placed in the urine-collection bottle. On awakening, void the urine and discard. From that time and during the next 24 hours collect the urine in the bottle containing the preservative. (This includes urine obtained upon awakening the second day.) If possible, keep the urine specimen refrigerated. When the 24 hour specimen has been collected, mix, measure its total volume and withdraw enough for the determinations.

Experiment 20. The Determination of Ammonia in Urine.* Permutit is a sodium aluminum silicate. The sodium may be reversibly replaced by other positive ions. Ammonia from urine replaces it in this method. The remainder of the urine is decanted and discarded. Upon the addition of strong sodium hydroxide solution, the ammonia from ammonium permutit is liberated. Nessler's solution, added directly, forms with ammonia a brownred compound which remains in solution for a short time. The color developed is a measure of the ammonia present.

PROCEDURE. Weigh, on a piece of filter paper, approximately 2 gm. of permutit.

Introduce the powder into a 200 ml. volumetric flask.

Add approximately 50 ml. of water. Shake and decant the supernatant liquid (which may contain some fine particles) and discard.

Add 4.5 ml. of water (no more), washing down the permutit from the neck of the flask.

Transfer 2 ml. of urine into the flask.

Rinse down the sides of the vessel with 1 to 5 ml. of water (no more).

Shake the flask gently for about five minutes.

Rinse the powder to the bottom of the flask with 25 to 40 ml. of water.

Allow the permutit to settle, and then decant and discard the supernatant liquid.

Repeat the process of adding 25 to 40 ml. of water and decanting twice more, being sure not to lose any permutit.

Add 5 ml. of water to the powder, and introduce into the flask 2 ml. of 10 per cent sodium hydroxide.

Shake the flask for a few minutes and then set it aside while preparing the standard ammonium sulfate solution.

Transfer 20.0 ml. of standard ammonium sulfate (1 ml. = 0.05 mg. *N*) to a clean 200 ml. volumetric flask.

Add 2 ml. of 10 per cent sodium hydroxide.

Dilute to about 150 ml. and shake.

Pour into a measuring cylinder 20 ml. of alkaline Nessler's reagent.

Swirl the volumetric flask (to set the solution spinning within the flask) and rapidly add the Nessler's solution. (A deep red but crystal-clear solution should result. If not, discard and prepare a fresh standard.)

* Folin and Bell, *J. Biol. Chem.*, *29*, 329 (1917).

Now dilute the contents of the flask containing the permutit and urina ammonia to about 150 ml.

Pour into a measuring cylinder, 20 ml. of alkaline Nessler's reagent.

Whirl the volumetric flask and add the Nessler's solution in the sa manner as in the case of the standard.

Dilute the contents of both flasks to 200 ml., stopper and invert seve times.

Compare in a colorimeter as soon as the permutit settles.

CALCULATIONS:

1. Calculate the number of milligrams of ammonia *N* per 100 ml. of uri
2. Calculate the quantity of ammonia in a 24 hour sample.

REAGENTS:

STOCK NESSLER'S SOLUTION. See Nonprotein Nitrogen (p. 81).

STANDARD AMMONIUM SULFATE SOLUTION. See Nonprotein Nitrog (p. 82).

10 PER CENT SODIUM HYDROXIDE. Dilute 200 ml. of 50 per cent sodiu hydroxide to 1 liter.

PERMUTIT. According to Otto Folin. (Obtained from Permutit Co.

(*a*) Derive the formula for calculation of the results.
(*b*) Give the function of permutit.
(*c*) What is meant by "elution"? Give several examples of its use.
(*d*) Suggest another method for the determination of urinary ammoni

Experiment 21. Determination of Phosphate in Urine.* Pipett 1.0 ml. of urine into a 100 ml. volumetric flask. Add approximately 70 m water. Pipette 5.0 ml. of a standard phosphate solution, containing 0.4 mg phosphorus per 5 ml., into another 100 ml. volumetric flask together wit some 65 ml. of water.

To each flask add 10 ml. of the molybdate reagent. Mix. To each add 4 m of the aminonaphthol sulfonic acid reagent. Mix and dilute with water to th 100 ml. mark. Invert and compare in the colorimeter.

CALCULATION: (Visual colorimeter, see p. 67)

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 0.4 \times \text{vol. of urine collected in 24 hrs.} = \text{mg phosphate per 24 hour urine specimen}$$

REAGENTS: See p. 103 under phosphates in blood; use the molybdate solu tion which is made up in 5 *N* sulfuric acid.

Experiment 22. Determination of Creatinine in Urine. Pipett 1.0 ml. of urine into a 100 ml. volumetric flask. Into a second flask pipett 1.0 ml. of a standard creatinine solution containing 1.0 mg. creatinine per m To each flask add 20 ml. of 1 per cent picric acid from a graduated cylinde followed by 1.5 ml. of 10 per cent NaOH. Mix and allow to stand for 1 minutes before comparison in a colorimeter.

* Fiske and Subbarow, *J. Biol. Chem.*, *66*, 375 (1925).

CALCULATION: (Visual colorimeter, see p. 67)

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{volume of urine in 24 hrs.} = \text{mg. creatinine per 24 hours.}$$

REAGENTS:

Standard creatinine solution: Dissolve 1 gm. creatinine in one liter of 0.1 *N* HCl.

Picric acid: Dissolve 1 gm. of purified picric acid in 100 ml. water.

Sodium hydroxide: Prepare a 10 per cent solution.

Experiment 23. The Determination of Chlorides in Urine.* The sample is titrated with standard mercuric nitrate solution at the proper acidity in the presence of diphenylcarbazone as indicator. Chlorides present react with the added mercuric ions to form soluble but undissociated mercuric chloride. An excess of mercuric ions turns the indicator purple. The end-point is sharp and relatively stable.

PROCEDURE. Pipette 0.20 ml. of a 24 hour urine specimen into a 50 ml. Erlenmeyer flask and add 1.8 ml. of distilled water. Add 1 to 3 drops of 0.67 *N* sulfuric acid and mix well. Then add 0.2 ml. of diphenylcarbazone indicator and titrate to a purple end-point with the standardized mercuric nitrate solution. (It is preferable to add the mercuric nitrate from a microburette. The size of the drops should be such that 1 ml. equals about 100 drops.)

CALCULATION: Ml. standardized mercuric nitrate $\times$ 100 = milliequivalents chloride as sodium chloride.

REAGENTS:

DIPHENYLCARBAZONE INDICATOR. Dissolve 200 mg. in 95 per cent ethyl alcohol. Make up to 100 ml. in a volumetric flask. Store in a dark bottle, preferably in a refrigerator. Prepare fresh monthly.

0.67 *N* SULFURIC ACID. Add 35 gm. of concentrated sulfuric acid to 1 liter of distilled water. Standardize against alkali of known strength.

MERCURIC NITRATE SOLUTION. In a liter volumetric flask dissolve 3 gm. of anhydrous C.P. mercuric nitrate in about 500 ml. distilled water, and add 2.5 ml. of concentrated nitric acid. When dissolved, make up to 1 liter. Mix, allow to stand a few days, then standardize. Dilute so that 1 ml. is equal to 100 milliequivalents of sodium chloride.

STANDARDIZATION OF MERCURIC NITRATE SOLUTION: (Carried out in triplicate). To 0.20 ml. of 0.1000 *N* sodium chloride solution in a 50 ml. Erlenmeyer flask add 1.8 ml. of distilled water, 3 drops of 0.67 *N* sulfuric acid and 0.2 ml. of indicator solution. Titrate with the stock mercuric nitrate to a purple end-point. Adjust so that 1 ml. of mercuric nitrate solution is equal to 100 milliequivalents of sodium chloride.

(*a*) Why is it important to keep the indicator cool and away from light?

(*b*) Why is it best that the *p*H of the sample be between 4.5 to 6.0 at the

* Schales and Schales, *J. Biol. Chem.*, *140*, 879 (1941); Asper, Schales and Schales, *J. Biol. Chem.*, *168*, 779 (1947).

beginning of the titration? (See reference to Asper, Schales and Schales.

(*c*) What is the origin of urinary chlorides?

(*d*) Can this "mercurimetric determination" be applied to blood seru and blood filtrates?

(*e*) Formulate the reaction between a solution of mercuric nitrate and solution of sodium chloride.

Chapter 14 Animal Experiments

Experiment 1. Effect of Insulin on Blood Glucose. Obtain a normal ood sample from a rabbit by a careful cardiac puncture, using heparin in ıe syringe and a No. 20 gauge needle.

Inject the animal subcutaneously with 4 units of insulin per kilogram of ɔdy weight.

At thirty minutes, one hour and one and one-half hours, obtain blood ımples.

Determine the glucose content of the various blood samples, using the olin-Wu, Shaffer-Hartmann or Nelson methods (pages 85, 87, 88).

Plot your results.

(*a*) What is the effect of insulin on blood glucose?

(*b*) If the rabbit were to get an overdose of insulin, what would you give to counteract this effect?

Experiment 2. Effect of Epinephrine on Blood Glucose. Follow the irections of Experiment 1, but inject 0.1 mg. of epinephrine per kilogram f body weight.

(*a*) What is the effect of epinephrine on the blood glucose?

(*b*) Where does the extra glucose come from?

(*c*) What other action has epinephrine?

Experiment 3. Levels of Free and Total Sulfanilamide in the Blood. repare a solution of a soluble sulfa drug, such as sodium sulfadiazine, or eutralize sulfanilamide with dilute sodium hydroxide. Inject 0.5 to 1 gm. f the drug intramuscularly.

At thirty minutes, one hour and one and one-half hours, withdraw blood amples as in Experiment 1.

Determine the free and total sulfa in the blood samples (p. 100).

Calculate the conjugated sulfa from your data and plot free and conjugated ulfa content of the blood against time.

(*a*) What is the formula for the sulfa compound that you injected?

(*b*) What sulfa compound will you use for construction of your standard urve?

(*c*) What is the chemical nature of the conjugated sulfa compound?

(*d*) Of what significance is the sulfa content of the blood during treatment y sulfa therapy?

Experiment 4. Effect of Impaired Kidney Function.* Twenty-four ours before the class meets, a rabbit is injected with sodium tetrathionate, 00 mg. per kilogram of body weight (see Appendix).

* Gilman, et al., *Am. J. Physiol., 147,* 115 (1946).

After twenty-four hours a complete anuria will result. Withdraw blood a perform the determination of all blood constituents normally done. T rabbit must be sacrificed after the completion of the experiment.

(*a*) Which blood constituents will have altered from the normal valu
(*b*) Why?
(*c*) In what diseases will similar findings be obtained?

Experiment 5. Determination of Rat Liver Glycogen. (This experim may be performed on two rats, one fasted for 24 hours, the other no Anesthetize a rat by injecting it intramuscularly with 5 mg. Nembutal per 1 gm. of rat. When the rat is under deep anesthesia, cut open the abdomi cavity and quickly cut a small piece (about 200 to 400 mg.) of the edge the liver. Immediately transfer this piece of liver to a 15 ml. gradua centrifuge tube containing 2 ml. of 30 per cent KOH which had been weigh carefully beforehand. Speed is essential since glycogen is lost very rapidly anaerobic glycolysis is allowed to proceed for any length of time. Weigh tube with the liver to get the weight of wet liver.

Heat the centrifuge tube containing the liver and KOH in a boiling wa bath for 30 minutes with gentle stirring. Cool in a beaker of cold water. A 2 drops of 10 per cent zinc sulfate solution and 4 ml. of 95 per cent ethan Stir and heat to boiling in the water bath. Cool and centrifuge. Discard t supernatant. Dissolve the precipitate in 2 ml. water and re-precipitate wi 4 ml. ethanol. Heat to boiling as before, cool, centrifuge and discard t supernatant.

Dissolve the precipitated glycogen in 2 ml. of 2.5 *N* sulfuric acid a heat in the boiling water bath for 30 minutes. Cool, add 1 drop of phen phthalein and 2.5 *N* NaOH, drop by drop with stirring, until the first pi color is obtained. Just discharge the color with a drop of 0.5 *N* sulfuric aci Dilute to 10 ml. with water, and mix.

Remove an aliquot of the solution for the determination of glucose (E periment 4, p. 85). Use 1 ml. aliquot if the liver is from a fasted rat and ml. of a 10 times diluted aliquot if the liver is from a fed rat. Calculate t glycogen content in terms of per cent glycogen of wet weight liver.

Experiment 6. Penetration of Radioactive Inorganic Phospha ($P^{32}O_4^{\equiv}$) into Red Cells. The instructor will provide the necessary i formation and demonstrations in order to acquaint the student with t problems involved in handling and measuring radioactive compounds. Th should include: nature of health hazards, monitoring devices, long distan manipulation technics and measuring instruments.

1. Remove 12 to 15 ml. of blood from a rabbit using heparin and mine oil. Transfer 5 ml. of blood to a 25 ml. Erlenmeyer flask under oil.

2. Add to this blood a small volume (0.1 to 0.5 ml.) of the radioacti phosphate solution containing 100,000 to 500,000 counts per minute. T actual activity will depend on the nature of the counting device; dipping tu for solutions, mica end-window tube for plated solids or a flow gas coun for greatest efficiency of counting.

3. Add the same volume of radioactive solution to another 5 ml. sample blood in another flask. This will serve as a control.

4. At 30, 60, 90 and 120 minutes, remove 0.1 ml. of blood under oil from e paraffin lined flask which is kept gently shaking throughout the incubation eriod. Add each sample to a graduated centrifuge tube containing 10 ml. of 9 per cent saline under oil. Mix and centrifuge. Remove the supernatant uid by means of a capillary tube connected to the suction pump with a large ask as a trap. Add 10 ml. of fresh saline to the cells, stopper, invert several mes and centrifuge again. Repeat twice again.

5. After the final centrifugation, add water to the cells up to a volume of ml. Shake to get a uniform solution. Withdraw aliquots of 1 ml. into ietal planchets. Add 1-2 drops of a detergent solution (Vel) and dry under n infra-red lamp.

6. Withdraw 0.1 ml. of the well-mixed control sample to a planchet and eat as above.

7. Measure the radioactivity of each sample.

8. Calculate and plot a graph to illustrate the extent and rate of incor-oration of inorganic phosphate into red cells.

(*a*) What errors are involved in this determination?

(*b*) How could these be overcome?

(*c*) In what respect is this method superior to the alternate method sing added inorganic phosphate in amounts which will allow the chemical letermination of differences?

Chapter 15 **Vitamins**

Experiment 1. Bioassay. Effect of Lack of Vitamin A on Grow This experiment is designed to acquaint the student with the fundamentals animal-feeding experiments. Many variations are possible and may be fou in the biochemical literature.

This experiment will be carried out cooperatively by a class of abo fifteen students during the course of a semester's work in general b chemistry. The class is divided into three groups; one is on duty on Monda another on Wednesday and the third on Friday. Each group is responsib for feeding and weighing the rats and cleaning their cages.

The basal diet, lacking in Vitamin A, can be prepared according to vario formulae such as the U.S.P., or may be conveniently purchased from t General Biochemicals, Inc., Chagrin Falls, Ohio. Rats weighing approximate 40 gm. are separated into two groups of six rats each. Group A receives t basal diet and water *ad lib.* Group B receives the same as group A plus drops cod liver oil orally administered by a medicine dropper. The avera weight of each group of animals is plotted against the days of the duratio of the experiment.

When the growth of rats in group A has slowed down to practically a ha eye symptoms may appear. This period corresponds to the depletion perio of the stored vitamin A. At this time group A is divided into two groups three rats each, group A1 and A2. Group A1 is continued as before and wi die after a period of decrease of weight. Group A2 is given the basal die but in addition, 2 drops of a solution of crystalline beta carotene in cotto seed oil, equivalent to 5 micrograms at each feeding. Group B is also divide into two groups of three rats each; B1 and B2. Group B1 is continued before with cod liver oil as a supplement, whereas group B2 is taken off th cod liver oil supplement. After a suitable length of time, the group still gettin the cod liver oil will pass the other group in average weight.

(*a*) Plot your results graphically.
(*b*) Where is vitamin A stored?
(*c*) What other source of vitamin A could be used?
(*d*) What variations in the experiment could you suggest?

Experiment 2. Chemical Assay of Ascorbic Acid. Dilute 1.0 ml. of standard solution of ascorbic acid (1.0 mg. per ml. of metaphosphoric acid acetic acid mixture) with 10 ml. of the metaphosphoric acid-acetic acid mix ture in a small Erlenmeyer flask or large test tube. Titrate with a solution o 2,6-dichlorphenol indophenol until a pink color is obtained which persist for 5 seconds. Compare with a water blank to which has been added 1 dro of the indicator.

Determine the ascorbic acid content of fresh orange juice, frozen orange juice (or other juices), homogenates (filtered) of apples and lettuce.

Squeeze the juice from one-half of an orange and strain. Mix 10 ml. of the juice with 10 ml. of the acid mixture. Titrate with the indicator.

For frozen orange juice, reconstitute according to the directions on the can. For apples and lettuce, homogenize in a Waring blender with an equal weight of the acid mixture, filter and titrate 10 ml. aliquots as above.

Reagents. See Appendix, p. 146.

Chapter 16 Laboratory Schedule for a Two-Semester Course in Biochemistry

THE FOLLOWING schedules represent the authors' experience with college seniors in a two-semester course in general biochemistry in the department of chemistry at The City College of New York. During the first semester the student spends 4 consecutive hours per week in the laboratory for a period of about 15 weeks. The minimum prerequisites are: one year general college chemistry, one-half year qualitative analysis and one-half year organic chemistry. During the second semester the student spends 5 consecutive hours each week in the laboratory. In addition to the prerequisites listed above the student will have taken another one-half year organic chemistry, and one-half year quantitative analysis. Most students will have had one year of physical chemistry.

FIRST SEMESTER BIOCHEMISTRY

(*Note:* For the quantitative colorimetric analyses, the students will use the visual colorimeters; see p. 67.)

PERIOD	LABORATORY ASSIGNMENT
1	(*a*) Check equipment. (*b*) Preparation of starch from potatoes. Exp. 12, p. 4. Save starch for period 2. (*c*) Tests for carbohydrates; use 1 per cent solutions of glucose, fructose, galactose, maltose, lactose, sucrose and starch: 1. Osazone. Exp. 8, p. 3. 2. Molisch. Exp. 1, p. 2. 3. Anthrone. Exp. 2, p. 2. 4. Iodine. Exp. 10, p. 4. 5. Benedict. Exp. 4, p. 2. 6. Seliwanoff. Exp. 6, p. 3.
2	The time-course of starch hydrolysis. Exp. 13, p. 5. Use the starch prepared during period 1. Save the remainder for period 6.
3-4	(*a*) Separation of the lipids in egg yolk. Exp. 1, p. 11. Preserve the egg white with a few drops of toluene in the refrigerator for period 5. Perform the following: (*b*) Phospholipids. Exp. 2, p. 12. (*c*) Glycerol. Exp. 3, p. 12.

ERIOD	LABORATORY ASSIGNMENT
	(*d*) Cholesterol. Exp. 10, p. 13. (*e*) Test for P. Exp. 12, p. 13. (*f*) Pancreatic lipase. Exp. 10, p. 60.
5	(*a*) Preparation of casein from milk. Exp. 2, p. 52. (*b*) Hydrolysis of casein. Exp. 28, p. 29. (Continue at period 6.) (*c*) Preparation of tyrosine by enzymatic hydrolysis of casein. Exp. 25, p. 26. (Continue to period 6.) (*d*) Determination of the protein content of egg white. Exp. 29, p. 29. (*e*) Protein tests; use the casein which you prepared as well as the egg white: 1. Millon's. Exp. 1, p. 18. 2. Hopkins-Cole. Exp. 3, p. 19. 3. Biuret. Exp. 4, p. 19. 4. Ninhydrin. Exp. 5, p. 19.
6	(*a*) Isolation of tyrosine from casein hydrolysate. Continue Exp. 25, p. 26. (*b*) Determination of tyrosine in casein hydrolysate. Exp. 28, p. 29. (*c*) Dialysis of colloids and crystalloids; dialyze a solution of a mixture of casein, glucose, glycine, sodium chloride and starch against distilled water. Test the outside solution for each of the compounds. (*d*) Cysteine. Exp. 7, p. 19. (*e*) Cystine. Exp. 6, p. 19. (*f*) Conversion of cystine to cysteine. Exp. 8, p. 20.
7	(*a*) Preparation and tests of ribose nucleic acid from yeast. Exp. 27, p. 28. (*b*) Finish isolation of tyrosine. Exp. 25, p. 26. (*c*) Buffers. Exp. 22, p. 25. (*d*) Test for N and P in casein. Exp. 12, p. 13, and Exp. 18, p. 24.
8	(*a*) Formol titration. Exp. 31, p. 31. (*b*) Donnan membrane equilibrium. Exp. 21, p. 24. (*c*) Demonstration of trypsin activity. Exp. 5, p. 59.
9	(*a*) Paper chromatography of amino acids. Exp. 10, p. 20. (*b*) Estimation of pepsin activity. Exp. 9, p. 42. (*c*) Denaturation and coagulation. Exp. 16, p. 23.
10	(*a*) Oxidase activity of milk. Exp. 6, p. 40. (*b*) Catalase activity. Exp. 8, p. 42. (*c*) Effect of calcium on milk clotting. Exp. 6, p. 53.
11	(*a*) Preparation of hemin. Exp. 2, p. 64.

PERIOD	LABORATORY ASSIGNMENT
	(*b*) Benzidine test for blood. Exp. 3, p. 64.
	(*c*) Spectroscopic examination of blood. Exp. 7, p. 6
	(*d*) Determination of hemoglobin (iron) in blood. Exp 20, pp. 121-123.
12	(*a*) Determination of non-protein nitrogen (NPN) blood. Exp. 2, p. 80.
	(*b*) Determination of serum alkaline phosphatase. Ex 14, p. 104.
	Note: for period 13, bring with you a 24-hour uri specimen. Exp. 19, p. 133.
13	(*a*) Determination of creatinine in urine. Exp. 22, p. 13
	(*b*) Determination of phosphate in urine. Exp. 21, p. 13
	(*c*) Determination of chlorides in urine. Exp. 23, p. 13
14	(*a*) Determination of the ascorbic acid content of food Exp. 2, p. 140.
	(*b*) Yeast fermentation (glycolysis). Exp. 10, p. 44.
15	Wash and dry glassware; check equipment.
	Note: During the course of this semester the student take part in a cooperative experiment illustratin the effect of vitamin A deficiency in rats. Exp. p. 140.

SECOND SEMESTER BIOCHEMISTRY

Because of the fact that the experiments performed during this semeste require some specialized and costly equipment, the class is divided int groups of 2, 3 or 4 students who work as a team on the same experiment Each student will do his own work except where advised otherwise. Thu on any one day, several different experiments will be going on in th laboratory. The laboratory is provided with 2 rabbits and 12 rats; th rabbits are used for blood samples and the rats for tissue samples. Fo colorimetric analyses students will use photocolorimeters.

PERIOD	LABORATORY ASSIGNMENT
A (first period for all)	Check equipment. Determination of plasma phosphate. Exp. 13, p. 102. Dur ing this experiment the students will learn to operate al types of colorimeters in the laboratory (see pp. 70-76).
B	Determination of calcium in plasma. Exp. 15, p. 106
C	Determination of carbon dioxide capacity of plasma Exp. 17, p. 111. Determination of oxygen capacity of whole blood. Exp 18, p. 115.
D	Formol titration of an amino acid using the glass elec trode. Exps. 33, 34, pp. 34-37.

PERIOD	LABORATORY ASSIGNMENT
	Glycogen content of rat liver; fed vs. fasted rats. Exp. 5, p. 138 and Exp. 4, p. 85. Remove food (not water) from rat to be fasted 18-24 hrs. before the experiment.
	Effect of insulin on blood glucose in the rabbit. Exp. 1, p. 137, Exp. 5, p. 87.
	Effect of epinephrine on blood glucose in the rabbit. Exp. 2, p. 137, Exp. 6, p. 88.
	Blood level of free and conjugated sulfadiazine in the rabbit. Exp. 3, p. 137.
	Absorption spectra of hemoglobin and its derivatives. Exp. 1, p. 76.
	Penetration of inorganic phosphate (P^{32}) into the rabbit red cell. Exp. 6, p. 138.
	Respiration of rat liver slices in the Barcroft-Warburg apparatus. Exp. 11, p. 45.
	Nonprotein nitrogen of rabbit blood by the micro Kjeldahl procedure. Exp. 3, p. 82.
	Total protein, albumin and globulin in rabbit plasma. Exps. 21, 22, pp. 123-126.
	Separation of carotenoids by chromatographic adsorption. Exp. 15, p. 16.
(last period for all)	Wash and dry apparatus; check equipment.

Appendix

PREPARATION OF STANDARD ACID AND ALKALI

Hydrochloric Acid, approximately 0.1 *N.* Dilute 8.5 ml. of concentrate hydrochloric acid to 1 liter with distilled water in a volumetric flask. Inve several times and transfer to a clean, dry bottle.

Dry approximately 5 gm. of pure anhydrous sodium carbonate on a watc glass in the oven at 110°C. for an hour.

Accurately weigh two charges of approximately 0.24 gm. of the dry sodiu carbonate, and transfer quantitatively to a 100 ml. beaker. Add 20 ml. c water in order to dissolve the carbonate.

Fill a clean, dry burette with the hydrochloric acid solution and titrate th carbonate solutions, using 1 drop of methyl red solution as an indicator. Jus before the end point is reached, boil the solution to expel the carbon dioxide and finish the titration when the solution is cool.

Calculate the exact normality of the hydrochloric acid solution and labe the solution appropriately.

Sodium Hydroxide, approximately 0.1 *N.* Prepare a saturated solution o sodium hydroxide and allow to stand for several days so that the sodium carbonate settles.

By means of a pipette, withdraw 5.3 ml. of the clear supernatant liquid an transfer to a 1 liter rubber-stoppered bottle. Dilute to approximately 1 lite with distilled water.

Using a pipette, transfer 20 ml. portions of the standard hydrochloric aci to 150 ml. beakers. Add 25 ml. of water and 1 drop of phenolphthalein solu tion to each. Titrate with the sodium hydroxide solution to a faint pink color

Calculate the normality of the sodium hydroxide solution and label th bottle accordingly.

APPROXIMATE CONCENTRATIONS OF LABORATORY REAGENTS

Acetic acid, glacial	17 *M*
Ammonium hydroxide, concentrated	18 *M*
Hydrochloric acid, concentrated	12 *M*
Nitric acid, concentrated	15 *M*
Sulfuric acid, concentrated	18 *M*

SOLUTIONS AND REAGENTS

Acetic Acid 5 *M,* 2 *M,* 1 *M,* 0.1 *M,* 0.01 *M.* Dilute 294 ml., 117 ml. 58 ml., 5.8 ml. and 0.58 ml. of glacial acetic acid to 1 liter with water t obtain each solution.

Acetate Buffer, 1 *M*, *pH* 4.7. Add 91 ml. of 1 *M* acetic acid solution to 109 ml. of 1 *M* sodium acetate solution. Check with a *p*H meter if available and adjust with the appropriate solution if necessary.

Albumin-Iodoeosin Powder. Separate the white of an egg from the yolk. Dilute the egg white four times with water and add the iodoeosin in the form of a finely ground suspension in a few milliliters of water. Heat the mixture to 80° to 90°C. with stirring to coagulate the protein. The colored coagulum is filtered by suction and air-dried on a porous plate overnight. The material is powdered and washed by shaking several times with 0.3 per cent hydrochloric acid solution and filtering. Wash with water and dry. Grind to a fine powder.

Ammonium Molybdate. Dissolve 100 gm. of molybdic acid in 144 ml. of concentrated ammonium hydroxide and 271 ml. of water. Slowly, and with constant stirring, pour the solution thus obtained into 489 ml. of concentrated nitric acid and 1148 ml. of water. Store in a glass-stoppered bottle.

Ammonium Oxalate, 0.2 *M*. Dissolve 28.4 gm. of ammonium oxalate monohydrate, in water to make 1 liter of solution.

Ammonium Sulfate, Saturated. Add about 77 gm. of ammonium sulfate to 100 ml. of water and shake.

Amyl Alcohol-Hydrochloric Acid Mixture for the Babcock Determination. Mix equal volumes of concentrated hydrochloric acid and amyl alcohol.

Anthrone Reagent, 0.2 per cent. Add 0.2 gm. of anthrone to 100 ml. of concentrated sulfuric acid and shake. The solution should be freshly prepared. (For preparation of anthrone, see *Organic Syntheses*, Collective Volume I, p. 60, N.Y. 1941.)

Arsenious Acid, 0.1 *M*. Dissolve 19.7 gm. of arsenious oxide in 100 ml. of 5 *M* sodium hydroxide solution. Add 2 *M* hydrochloric acid solution until the red color of phenolphthalein in the solution is just discharged. Dilute to 1 liter with water.

Barfoed's Reagent. Dissolve 13.3 gm. of crystallized copper acetate in 200 ml. of water. Filter, if necessary, and add 1.9 ml. of glacial acetic acid.

Barium Chloride, 0.5 *M*. Dissolve 104 gm. of barium chloride in water to make 1 liter.

Barium Hydroxide, saturated solution. Add about 40 gm. of barium hydroxide to 1 liter of water and shake.

Benedict's Qualitative Reagent. Dissolve 173 gm. of crystalline sodium citrate and 100 gm. of anhydrous sodium carbonate in about 800 ml. of water. Stir thoroughly and filter. Add to the filtered solution 17.3 gm. of copper sulfate dissolved in 100 ml. of water. Make up to 1 liter with distilled water.

Benzidine, saturated solution. Dissolve enough benzidine in glacial acetic acid to make a saturated solution.

Biuret Reagent. Prepare two solutions: (*a*) 0.01 *M* copper sulfate. Dissolve 2.5 gm. of copper sulfate in 1 liter of water. (*b*) 10 *M* sodium hydroxide. Dissolve 440 gm. of sodium hydroxide in sufficient water, and make up to 1 liter.

Boric Acid, 0.5 *M*. Dissolve 31 gm. of boric acid in about 900 ml. of water. Make up to 1 liter with water.

Bromphenol Blue, 0.04 per cent. Grind 0.1 gm. of the dry powder in a

mortar with 14.9 ml. of 0.01 *M* sodium hydroxide. Dilute to 250 ml. w distilled water.

Bromthymol Blue, 0.04 per cent. Grind 0.1 gm. of the dry powder i mortar with 16 ml. of 0.01 *M* sodium hydroxide. Dilute to 250 ml. w distilled water.

Bromide Water, saturated solution. Shake 100 ml. of water with liqu bromine until no more bromine dissolves.

Buffer Solutions (from *p*H 5.3 to 8). Prepare the following solutio (*a*) Disodium hydrogen phosphate, *M*/15. Dissolve 23.88 gm. $Na_2HPO_4.12H_2O$ in water to make 1 liter of solution. (*b*) Potassium di drogen phosphate, *M*/15. Dissolve 9.08 gm. of KH_2PO_4 in water to ma 1 liter of solution.

BUFFER SOLUTIONS

A. Phosphate Buffers:

*p*H	*M*/15 Na_2HPO_4 Ml.	*M*/15 KH_2PO_4 Ml.
5.4	3.0	97.0
5.6	5.0	95.0
5.8	7.8	92.2
6.0	12.0	88.0
6.2	18.5	81.5
6.4	26.5	73.5
6.6	37.5	62.5
6.8	50.0	50.0
7.0	61.1	38.9
7.2	71.5	28.5
7.4	80.4	19.6
7.6	86.8	13.2
7.8	91.4	8.6
8.0	94.5	5.5

B. Acetate Buffers:

*p*H	*Ml. 0.2 M Sodium Acetate*	*Ml. 0.2 M Acetic Acid*
3.6	15	185
3.8	24	176
4.0	36	164
4.2	53	147
4.4	74	126
4.6	98	102
4.8	120	80
5.0	141	59
5.2	158	42
5.4	171	29
5.6	181	19

Cadmium Chloride, alcoholic. Prepare a saturated solution of cadmiu chloride in 95 per cent ethyl alcohol.

Calcium Chloride, 0.5 *M*. Dissolve 55.5 gm. of dry calcium chloride i water to make 1 liter of solution.

Catechol, 0.2 per cent. Dissolve 0.2 gm. of catechol in 100 ml. of wate

Copper Sulfate Solution, 0.01 *M*. Dissolve 0.25 gm. of the salt (blu vitriol) in 100 ml. of water.

2,6,-Dichlorphenol Indophenol. 84 mg. of sodium bicarbonate and 104 mg. of the sodium salt of the indicator are dissolved in 200 ml. water. Filter if necessary and store in refrigerator.

Digitonin Solution, 1 per cent in 80 per cent alcohol. Dissolve 1 gm. of digitonin in 85 ml. of 95 per cent alcohol. Add 15 ml. of water and mix well. It may be necessary to filter the solution before use.

2,4 Dinitrophenylhydrazine Solution, saturated in 2 *N* HCl. Heat on the steam bath 1 gm. of the compound in 100 ml. of 2 *N* hydrochloric acid until it is all dissolved. Cool and filter before use.

Ehrlich's Diazo Reagent. Prepare two solutions and keep separate until needed; (*a*) 0.5 per cent solution of sodium nitrite in water (0.5 gm. per 100 ml. of water), and (*b*) add 0.5 gm. of sulfanilic acid and 5 ml. of concentrated hydrochloric acid to 100 ml. of water. Mix just before use in the proportion of 1 part (*a*) to 50 parts (*b*).

Ferric Chloride, 0.1 *M.* Dissolve 27 gm. of hydrated ferric chloride in water to make 1 liter of solution.

Folin Phenol Reagent. Reflux a mixture of 100 gm. sodium tungstate, 25 gm. sodium molybdate, 700 ml. water, 50 ml. of 85 per cent phosphoric acid and 100 ml. of concentrated HCl for 10 hours. Add 150 gm. lithium sulfate, 50 ml. water and 3 drops of bromine. Boil until the excess bromine is removed. Cool. Dilute to one liter with water and filter. Store in a brown bottle.

Formaldehyde, 0.5 per cent. Dilute 10 ml. of approximately 40 per cent formaldehyde solution with 800 ml. of water. Adjust the *p*H to 7.

Fructose, 1 per cent. Dissolve 10 gm. of fructose in 1 liter of water. Preserve with toluene.

"Fusion Mixture." Mix intimately 3 parts of anhydrous sodium carbonate with 2 parts of potassium nitrate.

Galactose Solution, 1 per cent. Dissolve 10 gm. of galactose in 1 liter of water. Preserve with toluene.

Gelatin Solution, 2 per cent. Warm 100 ml. of water to 65° to 70°C. and dissolve 2 gm. of gelatin. Keep at room temperature, but not lower; otherwise it gels.

Glucose Solution, 1 per cent. Dissolve 10 gm. of glucose in 1 liter of water. Preserve with toluene.

Hopkins-Cole Reagent. Place 10 gm. of powdered magnesium in a large Erlenmeyer flask. Add distilled water until the magnesium is liberally covered. Slowly pour 250 ml. of a cold saturated solution of oxalic acid into the flask, shaking all the while (cool under running water, if necessary). Filter. Just acidify the filtrate with acetic acid and dilute to 1 liter.

Hydrochloric Acid, M. Add 1 part of concentrated hydrochloric acid to 11 parts of water.

Iodine Solution, 0.01 *M.* Dissolve 10 gm. of potassium iodide in 1 liter of water. Add 2.5 gm. of iodine to this solution and stir.

Lactose Solution, 1 per cent. Dissolve 10 gm. of lactose in 1 liter of water. Preserve with toluene.

Lead Acetate Solution, 0.2 *M.* Dissolve 76 gm. of hydrated lead acetate, $Pb(C_2H_3O_2)_2.3H_2O$, in water to make 1 liter of solution.

Lead Nitrate Solution, 0.5 *M.* Dissolve 16.5 gm. of lead nitrate in 1(
ml. of water.

Magnesia Mixture. Dissolve 175 gm. of hydrated magnesium sulfa
and 350 gm. of ammonium chloride in 1400 ml. of distilled water. Add 7
ml. of concentrated ammonium hydroxide, mix thoroughly, and store in
stoppered bottle.

Maltose Solution, 1 per cent. Dissolve 10 gm. of maltose in 1 liter
water. Preserve with toluene.

Mercuric Chloride, 0.2 *M.* Dissolve 54.3 gm. of mercuric chloride
water to make 1 liter of solution.

Mercuric Sulfate, 10 per cent in *M* sulfuric acid. Dissolve 10 gm.
mercuric sulfate in 100 ml. of *M* sulfuric acid.

Metaphosphoric-Acetic Acid Mixture. Mix 40 ml. glacial acetic aci
with 450 ml. water. Dissolve 15 gm. metaphosphoric acid in this mixtur
Filter and refrigerate.

Methyl Red Solution, 0.5 per cent. Dissolve 0.5 gm. of methyl red i
100 ml. of 95 per cent ethyl alcohol.

Methylene Blue-Methyl Red Mixed Indicator. This solution consists
1 part of 0.2 per cent methyl red in alcohol and 1 part of 0.1 per cer
methylene blue in alcohol. The *p*H change is at 5.4. The color is green i
alkaline solution (*p*H 5.6), colorless at *p*H 5.4 (end point) and reddish-pin
in acid solution (*p*H 5.2).

Methylene Blue, 0.02 per cent. Dissolve 0.2 gm. of "certified" methylen
blue in 1 liter of water.

Millon's Reagent. Dissolve 10 gm. of mercury in 20 ml. of concentrate
nitric acid. When solution is complete and the brown fumes cease to b
evolved, dilute with 60 ml. of water. Decant the supernatant liquid and stor
in a glass-stoppered bottle.

Molisch Reagent. Dissolve 10 gm. of α-naphthol in 100 ml. of 95 pe
cent ethyl alcohol.

Molybdate Solution. See *Ammonium molybdate.*

Ninhydrin (triketo-hydrindene hydrate), 0.1 per cent. Dissolve 0.1 gm
of the solid in 100 ml. of water.

Obermayer's Reagent. Dissolve 3 gm. of ferric chloride in 1 liter o
concentrated hydrochloric acid.

Oxalic Acid, 0.5 *M.* Dissolve 63 gm. of the dihydrate in water to mak
1 liter of solution.

Pepsin Solution, 0.1 per cent. Grind 1 gm. of pepsin with 50 ml. o
water in a mortar until it is a thin paste. Dilute to 1 liter with water.

Pepsin Solution, 0.2 per cent, in 0.1 *M hydrochloric acid.* Add 0.2 gm
of pepsin to 100 ml. of 0.1 *M* hydrochloric acid, and stir.

*p*H RANGES OF COMMON INDICATORS

Name	*p*H *Range*	*Color Change*
Acid cresol red	0.2– 1.8	Red-Yellow
Acid meta cresol purple	1.2– 2.8	Red-Yellow
Thymol blue	1.2– 2.8	Red-Yellow
Benzo yellow	2.4– 4.0	Red-Yellow

Name	*p*H *Range*	*Color Change*
Bromcresol green	3.8– 5.4	Yellow-Blue
Bromphenol blue	3.0– 4.6	Yellow-Blue
Methyl red	4.4– 6.0	Red-Yellow
Chlorphenol red	5.2– 6.8	Yellow-Red
Bromcresol purple	5.2– 6.8	Yellow-Purple
Bromthymol blue	6.0– 7.6	Yellow-Blue
Phenol red	6.8– 8.4	Yellow-Red
Cresol red	7.2– 8.8	Yellow-Red
Meta cresol purple	7.6– 9.2	Yellow-Purple
Thymol blue	8.0– 9.6	Yellow-Blue
Phthalein red	8.6–10.2	Yellow-Red
Tolyl red	10.0–11.6	Red-Yellow
Parazo orange	11.0–12.6	Yellow-Orange
Acyl blue	12.0–13.6	Red-Blue

Phenol Red, 0.04 per cent. Grind 0.1 gm. of the dry powder in a mortar with 28.2 ml. of 0.01 *M* sodium hydroxide. Dilute to 250 ml. with distilled water.

Phenolphthalein Solution, 0.5 per cent in alcohol. Dissolve 0.5 gm. of phenolphthalein in 100 ml. of 95 per cent alcohol.

Phenylhydrazine Mixture. Add 2 gm. of phenylhydrazine hydrochloride to 30 ml. of water and stir for several minutes; filter. If the compound is pure it will dissolve completely. Add 3 gm. of anhydrous sodium acetate and mix well. (Caution! Phenylhydrazine is poisonous. Avoid contact with skin!)

Phosphotungstic Acid. Dissolve 100 gm. of sodium tungstate and 70 gm. of disodium phosphate in 500 ml. of water. Add nitric acid to an acid reaction.

Picric Acid, saturated solution. Dissolve 1.2 gm. of the solid in 100 ml. of water, or allow distilled water to stand in contact with an excess of picric acid.

Potassium Chloride, M/75, in glacial acetic acid. Dissolve 0.1 gm. of potassium chloride in 100 ml. of glacial acetic acid.

Potassium Ferrocyanide, 0.2 *M*. Dissolve 84.5 gm. of the trihydrate in water to make 1 liter of solution.

Potassium Hydroxide, 10 per cent alcoholic solution. Dissolve 10 gm. of potassium hydroxide in 100 ml. of 95 per cent alcohol.

Potassium Iodide Solution, 15 per cent. Dissolve 15 gm. of potassium iodide in water to make 100 ml. of solution.

Preservative Solution. Dissolve 1 gm. of thymol in 100 ml. of toluene.

Pyrophosphate Solution 0.1 M, *p*H 8.3. To 200 ml. of 9 per cent sodium pyrophosphate solution add 16 ml. of 1 *N* hydrochloric acid and dilute to 400 ml. with water. Check the *p*H with a *p*H meter if one is available.

Rennin Solution, 0.1 per cent. Grind 1 gm. of rennin preparation with 50 ml. of water in a mortar until it is a thin paste. Dilute to 1 liter with water. For the 1 per cent solution use 10 gm. of rennin.

Seliwanoff's Reagent. Dissolve 0.05 gm. of resorcinol in 100 ml. of dilute hydrochloric acid (1 part of concentrated hydrochloric acid diluted with 2 parts of water).

Silver Nitrate, 0.1 *M.* Dissolve 17 gm. of silver nitrate in water make 1 liter of solution.

Sodium Alizarin Sulfonate. Dissolve 1 gm. in 100 ml. of water.

Sodium Bicarbonate, 5 per cent. Fifty grams of the salt dissolved in liter of water.

Sodium Carbonate, saturated solution. Add 238 gm. of the decahydra to 100 ml. of water and stir.

Sodium Carbonate, 1 *M.* Dissolve 286.2 gm. of the decahydrate or 1(gm. of the anhydrous salt in water, and dilute to 1 liter.

Sodium Chloride, isotonic solution. Dissolve 0.9 gm. of sodium chlori in 100 ml. of water.

Sodium Chloride, saturated solution. Add approximately 37 gm. to 1(ml. of water. If not saturated, continue the addition of the sodium chlorid

Sodium Chloride, 1.75 *M.* Dissolve 10.2 gm. of sodium chloride water to make 100 ml. of solution.

Sodium Chloride, 0.1 *M* solution. Dissolve 5.85 gm. in water to make liter of solution.

Sodium Hydroxide, saturated solution. Add in small portions abo 100 gm. of good quality sodium hydroxide to 100 ml. of distilled water in a Erlenmeyer flask. Stopper, and allow to stand for a few days or until th sodium carbonate settles to the bottom, leaving a clear solution of sodiu hydroxide, practically carbonate-free. This solution contains about 50 gm. sodium hydroxide per 100 ml. (approximately 50 per cent solution, or 12. *M*).

Sodium Hydroxide, approximately 0.1 *M.* Add 5.3 ml. of saturate sodium hydroxide solution to water to make 1 liter of solution.

Sodium Hydroxide, 1 *M.* Dissolve 40 gm. of sodium hydroxide in 20 ml. of water. Dilute to 1 liter with water.

Sodium Hydroxide, 2 *M.* Dissolve 80 gm. of sodium hydroxide i enough water to bring the volume to 1 liter.

Sodium Nitrite, 1 *M.* Dissolve 7 gm. in 100 ml. of water.

Sodium Nitroprusside, saturated solution in methanol. Saturate 10 ml. of methanol with powdered sodium nitroprusside.

Sodium Nitroprusside, 0.1 *M.* Dissolve 30 gm. of sodium nitroprussid in water to 1 liter.

Starch, 1 per cent. Mix 1 gm. of soluble starch with a little water, sti and add to 100 ml. of boiling water.

Stokes' Reagent. Add 2 gm. of ferrous sulfate and 3 gm. of tartari acid to 100 ml. of water. Stir. When ready for use, take a few milliliters in test tube and add concentrated ammonia water drop by drop until th precipitate which first forms is entirely dissolved.

Sucrose Solution, 0.1 *M.* Dissolve 34.2 gm. in water, and make up t 1 liter. Preserve with toluene.

Sucrose, 1 per cent. Dissolve 1 gm. of sucrose in 100 ml. of water.

Sulfuric Acid, approximately 0.1 *M.* Carefully add 5.5 ml. of con centrated sulfuric acid to water, cool, mix thoroughly and dilute to 1 liter

Tannic Acid, 10 per cent. Dissolve 10 gm. of tannic acid in 100 ml. of water.

Toluene-Thymol Solution (for preserving biological solutions). See *Preservative solution.*

Trichloracetic Acid, 30 per cent. Dissolve 300 gm. of trichloracetic acid in 700 ml. of water. Keep the solution in the refrigerator.

Urea, 0.1 *M.* Dissolve 6 gm. of urea in 1 liter of water.

Xanthydrol Solution in Methyl Alcohol (approximately 10 per cent). Suspend 10 gm. of xanthydrol in 90 gm. of absolute methyl alcohol (113 ml.). Shake vigorously. After two days filter through dry paper into a narrow-mouthed, well-stoppered amber bottle. (The xanthydrol may be purchased from Eastman Kodak Co.)

LOGARITHMS

No.	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	10	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	11	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6386	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9

LOGARITHMS

No.	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	2	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4
	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9

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